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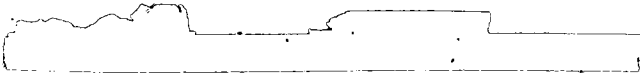
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POST-AMADORI REACTIONS OF SERUM PROTEINS

Sheila Cochrane BSc (Hons)

**Thesis submitted for the degree of Doctor of Philosophy in the
discipline of Biophysics**

**Open University
Oxford Research Unit
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ABSTRACT

The formation of glycofluorophores and the fragmentation of proteins is only seen if glucose (or another reducing sugar) is included in the incubation. Although similar changes have been reported in the absence of sugars, it has been shown here that these changes are glucose dependent. Contrary to previous work, I have shown that the presence of bound lipid on HSA does not alter the formation of Amadori product. Fragmentation of HSA is also unaffected by the presence of bound lipid, but the formation of fluorescence by native HSA is 2.5 times greater than that of delipidated HSA. This shows that (i) the enhanced fluorophore formation seen in native HSA is independent of the amount of Amadori product, (ii) that at least two components of fluorescence on native HSA exist and (iii) that the mechanisms of fragmentation and lipid-dependent fluorescence formation are not the same.

Both fragmentation and fluorescence formation were inhibited by the transition-metal chelator DTPA, showing that both these reactions are transition-metal catalysed. The formation of glycofluorophores appears to be partially dependent on the high affinity binding of transition metals to the protein, but the glucose-induced fragmentation of both albumin and human transferrin occurs only when the transition metal species present is copper. No fragmentation of the iron binding protein transferrin is seen when iron is included in the incubations in the place of copper. Both these reactions were also inhibited by the use of HEPES buffer in the place of phosphate buffer. HEPES buffer, unlike phosphate buffer, has free radical scavenging properties so therefore it would appear that in addition to being transition-metal catalysed, these reactions are free radical mediated processes. The carbonyl scavenger, aminoguanidine, inhibited the production of glycofluorophores implicating a carbonyl intermediate in this reaction. Pre-incubation of glucose with copper caused an increase in the amount of fluorescence initially observed indicating that autoxidation of glucose plays a role in fluorophore formation, although it is not possible to say whether this is a major reaction pathway or not.

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INTRODUCTION

1.1 Introduction

The spontaneous reaction between glucose and amino acids was first recorded by Maillard in 1910. Until the 1970's the Maillard reaction, which is characterised by the development of brown colour, fluorescence and crosslinking materials, was chiefly studied by food chemists [Bucala et al, 1991]. During the 1970's the chemistry of the Maillard reaction was found to occur *in vivo* and the measurement of early products of the reaction between reducing sugars and proteins became clinically important in the monitoring of diabetic long-term blood glucose control. There is now a large amount of evidence of the correlation between diabetic complications and high concentrations of blood sugar.

The first stage of the reaction is between a reducing sugar and an amino group on a protein; this produces an unstable aldimine, or Schiff's base. The Schiff's base forms rapidly, is very labile (its dissociation constant is nearly equal to its association constant) and reaches equilibrium with free glucose in a few hours [Baynes et al, 1984]. The Schiff's base can undergo the Amadori rearrangement to form a more stable ketoamine or Amadori product (see Figure 1.1). The formation of Amadori product is reversible, but *in vitro* it can take 3 weeks or more for equilibrium to be reached, the rate of the back reaction being dependent on reaction conditions [Baynes et al, 1984]. It has been found that in haemoglobin, the rate constants for the formation and dissociation are $14.2 \times 10^{-6} \text{s}^{-1}$ and $1.7 \times 10^{-6} \text{s}^{-1}$ respectively [Mortensen and Christopherson, 1983]. *In vivo* the concentration of Amadori product is determined by the glucose concentration and the half life of the protein. Therefore, the Amadori product of haemoglobin (HbA_{1c}) and human serum albumin (HSA) make them useful indicators of blood glucose control over a period of weeks [Schleicher and Wieland, 1989]. Although the term 'glycation' is sometimes used loosely to cover any sugar induced protein modification, it is restricted in this thesis to its correct usage, i.e. to describe the formation of Amadori product.

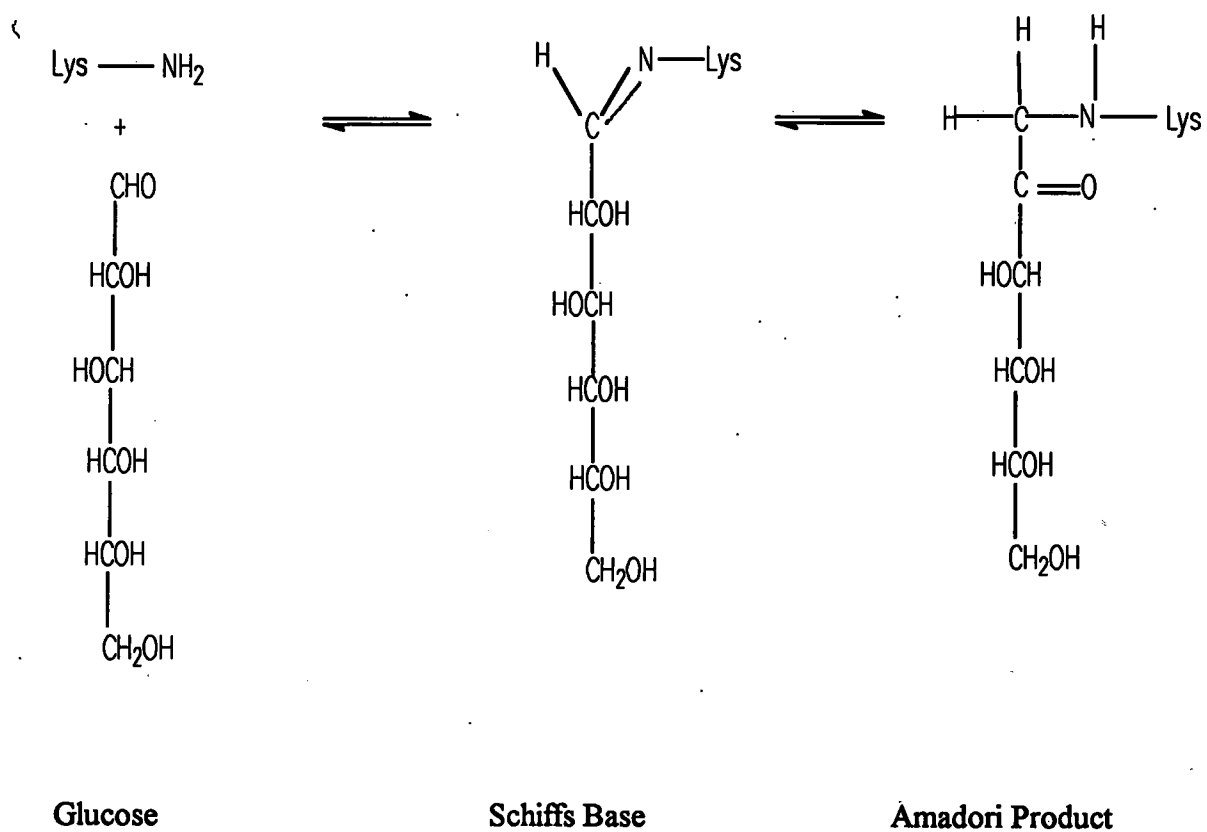


Figure 1.1: Formation of Amadori Product

1.2 Factors affecting glycation

In vitro glycation is affected by temperature, incubation time (at least up to the point where the steady state is reached), pH (the nucleophilic addition of the amino group to the electrophilic carbon atom of the carbonyl group is governed by acid-base catalysis), and the concentration of the reactants. *In vivo* the major variable is the concentration of reactants, hence the extent of *in vivo* glycation is determined by the concentration of blood glucose and the half-life of each protein [Schleicher and Wieland, 1986].

Glycation rates are also affected by the type of reducing sugar which is reacting with the protein. Bunn and Higgins (1981) investigated the covalent binding of radioactive sugars to haemoglobin using a variety of sugars. They found that with all the sugars the covalent binding to haemoglobin was linear with time. They also found a strong correlation between the rates of reactivity of the sugars with haemoglobin, and the percentage of each sugar found in the open chain form. It is necessary for the sugar to be in the acyclic form in order for the carbonyl group to exist. Two factors other than the percentage of sugar present in the acyclic form were found to affect the rate of the reaction between reducing sugar and haemoglobin; (i) ketoses are less reactive than aldoses (probably because an aldehyde carbonyl carbon atom is a stronger electrophile than a ketone carbonyl carbon), and (ii) the presence of charged groups on the sugar can influence its reactivity with haemoglobin.

Glucose is a relatively unreactive sugar and only 0.002% exists as the acyclic form. In contrast, fructose has 7.5 times greater reactivity with haemoglobin (despite being a ketose) and has 0.7% existing as the open chain form [Bunn and Higgins, 1981].

The formation of Amadori products from Schiff's base can be accelerated by acid-base catalysis. In HSA, the amino acid sequence around glycation sites was examined, and it was found that the lysine residues glycated *in vivo* often appear in a sequence with proton-donating amino acid residues. In HSA, Lys-525 is the principal site of *in vivo* glycation with

some glycation of lysine residues 439 and 534. Lys-525 is part of a Lys-Lys sequence, Lys-439 is part of a Lys-His sequence and Lys-530 is in a Lys-His-Lys sequence. Other lysine residues in HSA which are glycated *in vitro*, such as Lys-199, appear in the primary structure close to disulphide bridges, which may hold proton- donating residues from other sections of the chain close enough to lysine residues to act as catalysts of the Amadori rearrangement [Iberg and Flückiger, 1986].

1.3 Consequences of Amadori product formation

The formation of Amadori product can inactivate certain enzymes [Taniguchi et al, 1989] and can lead to conformational changes in lens α and γ crystallins [Beswick and Harding, 1987] and HSA [Shaklai et al, 1984]. The glycation-induced conformational change in HSA does not appear to affect its function *in vivo* [Murtiashaw and Winterhalter, 1986]. The glycation of lens crystallins results in loss of positive charges and leads to a change in conformation which allows previously unexposed thiol groups to participate in disulphide bonds [Beswick and Harding, 1987]. The Amadori product itself is reactive and can undergo many post-Amadori reactions which lead to compounds with the general name of Maillard products. There is increasing evidence that protein glycation alone (the formation of Amadori product) is not sufficient to cause the complications seen in diabetes. It has been found that there is no age-linked increase in the amount of Amadori product found in collagen of human skin or basement membranes, or in lens proteins of patients older than 5 years old [Baynes and Monnier, 1989]. A recent study by Scalbert and Birlouez-Aragon (1993) showed that the fluorescence of human lens proteins increased with age and cataracts, but the amount of Amadori product remained constant. This would appear to lend weight to the idea that it is not the accumulation of Amadori products that is the cause of diabetic complications, but the products of post-Amadori reactions. Therefore this thesis has concentrated on the formation of these products.

1.4 Post-Amadori Reactions

The products of post-Amadori reactions are known as advanced glycation endproducts, or AGEs, and include crosslinks, fluorophores, melanoids and peptide fragments resulting from nonenzymatic proteolysis. To date very few of these reactions or their products have been satisfactorily characterised. One of the commonest ways of monitoring AGE formation is to look at the build-up of fluorescence. This is one approach adopted in this thesis, using the wavelength characteristic of pentosidine (see Section 1.4.2). The other approach used here is to monitor protein fragmentation (see Section 1.8).

1.4.1 3-Deoxyglucosone

Food chemists first detected 3-deoxyglucosone as a degradation product of Amadori products refluxed under acidic conditions in the 1960s (see Figure 1.2) [Anet, 1964]. Kato et al (1987) found that 3-deoxyglucosone is produced under physiological conditions in *in vitro* Maillard reactions, and that 3-deoxyglucosone is the species responsible for the glucose-induced crosslinking of lysozyme *in vitro*. It was found that 3-deoxyglucosone was the major carbonyl compound in the low molecular weight fraction of incubation media in which either fructose or glucose was incubated with protein to form Amadori product [Shin et al, 1988]. The amount of 3-deoxyglucosone produced during *in vitro* protein glycation is dependent on the number of free lysine residues. The generation of 3-deoxyglucosone is suppressed if the lysine residues are blocked by acetylation. The production of 3-deoxyglucosone from fructose is unaffected by the availability of lysine residues implying that 3-deoxyglucosone can be formed from fructose independently of an Amadori intermediate [Shin et al, 1988].

In vitro 3-deoxyglucosone has been shown to be involved in the production of both crosslinks and the uncharacterised fluorescent product L1 and its role in the formation of

the well characterised AGE, pyrraline, has been postulated [Kato et al, 1989; Knecht et al, 1992]. The formation of AGEs from 3-deoxyglucosone can be inhibited by aminoguanidine. Studies by Igaki et al (1990) in which both 3-deoxyglucosone and Amadori products were treated with aminoguanidine showed a reduction in crosslink formation. This suggests that 3-deoxyglucosone is responsible for the crosslinks, and that the Maillard reaction proceeds through dicarbonyl intermediates such as 3-deoxyglucosone.

In vivo it is thought that 3-deoxyglucosone is formed from glucose either via its Amadori product (which rearranges to regenerate the free amino group and produce 3-deoxyglucosone), or via the polyol pathway in which glucose is converted to fructose which undergoes a rearrangement and a dehydration to give 3-deoxyglucosone [Knecht et al, 1992]. This may be of particular concern in diabetes owing to the increased activity of the polyol pathway [Baynes and Monnier, 1989]. It is thought that 3-deoxyglucosone is converted enzymatically to the less reactive species 3-deoxyfructose. Both of these compounds have been detected in human urine. This would suggest that the ability of an individual to convert reactive 3-deoxyglucosone to the less reactive 3-deoxyfructose could be a factor determining the extent of development of diabetic complications [Knecht et al, 1992]. Recent work by Niwa et al (1993) has shown that the serum levels of 3-deoxyglucosone are elevated in diabetics and especially so in those with nephropathy.

1.4.2 Pentosidine

Pentosidine is a fluorescent crosslink with excitation and emission maxima of 328nm and 378nm respectively (Figure 1.2). It is formed between lysine and arginine residues by a five-carbon sugar. It was characterised by Sell and Monnier (1989) after extraction from human dura mater collagen from diabetics and then synthesised *in vitro* by reacting lysine and arginine with D-ribose. At first, pentosidine could not be detected in the products from the reaction between lysine, arginine and glucose or any other hexose, so it was concluded that pentosidine must be formed by reaction of lysine and arginine with pentoses. However,

Grandhee and Monnier (1991) were able to detect the slow formation of pentosidine from BSA incubated with glucose, fructose or ascorbate. From further studies it was established that the Amadori products of glucose and fructose with lysine were in fact precursors of pentosidine and the pentose reaction product furfural was not a precursor of pentosidine, although pentoses could contribute to the formation of pentosidine through another route.

After *in vitro* glycation with glucose, pentosidine only makes up 0.001 moles of each mole of lysozyme dimer formed [Dyer et al, 1991a]. It is found to increase with both age and diabetes *in vivo* and is the only fluorescent AGE to be characterised so far. Its use as a biomarker for the Maillard reaction *in vivo* was postulated by Dyer et al (1991b). Sell and Monnier (1990) showed, in a study linking diabetic complications with pentosidine levels, that all the diabetics with elevated pentosidine levels had retinopathy. It has also been demonstrated that diabetics have elevated urinary levels of pentosidine, with an excretion of 75µg per day compared to 29µg per day in control patients [Takahashi et al, 1993]. In this study, glucose-induced fluorescence was monitored using the excitation and emission maxima of isolated pentosidine.

1.4.3 Furoyl-furanyl imidazole (FFI)

In 1984 Pongor et al identified an AGE that was believed to be formed from condensation of two Amadori products, but this was later shown to be an artefact of the extraction process [Njoroge et al, 1988].

1.4.4 Pyrraline

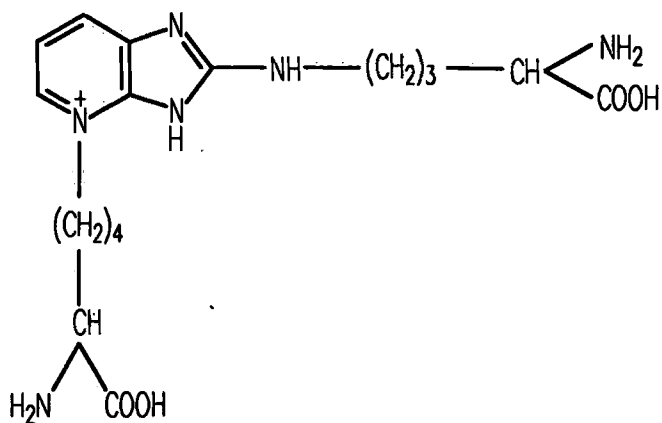
Pyrraline and other pyrroles are believed to be formed from the reaction between free amino groups on lysine residues and 3-deoxyglucosone. An ELISA was developed for pyrraline, as it is destroyed by acid hydrolysis so cannot be detected by most chemical methods.

Using this assay, Hayase et al (1989) showed that diabetics had elevated levels of pyrraline bound to HSA compared to normal patients.

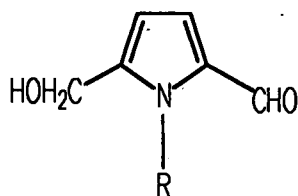
Another group of related compounds that have been formed *in vitro* from reactions of Amadori product with 3-deoxyglucosone are the AFGs (1-alkyl-2-formyl-3,4-diglycosyl pyrroles). These were isolated by using sulphites to inhibit the later reactions in the Maillard reaction to trap intermediates such as these compounds [Bucala et al, 1991]. The use of sulphites as inhibitors of the Maillard reaction is widespread in the food industry. The structures of pyrraline and AFG are shown in Figure 1.2.

1.4.5 CML

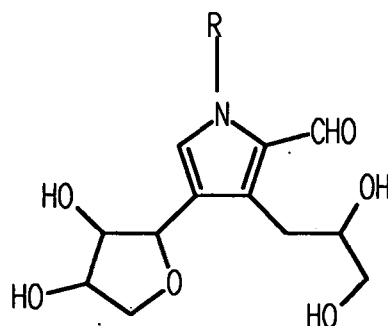
The Amadori product can undergo oxidative cleavages to form carboxymethyl lysine (CML) and erythronic acid (EA), or lysino-lactic acid (LL) and glyceric acid, depending on whether the cleavage occurs between C2 and C3 or C3 and C4. CML and EA are the major products in this reaction. CML and LL are both colourless and much less reactive than the Amadori product. Both CML and LL are found in urine and in hydrolysates of lens proteins [Ahmed et al, 1988; Ahmed et al, 1986].



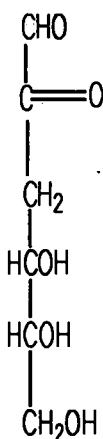
Pentosidine



Pyrraline



AFGP



3-Deoxyglucosone

Figure 1.2: Structures of 3-deoxyglucosone and the AGEs pentosidine, pyrraline and AFGP (1-alkyl-2-formyl-3,4-diglycosyl pyrroles)

1.5 The role of oxidation in AGE formation

Over the past few years, it has become clear that the severity of diabetic complications is related not to the build up of Amadori product, but to post-Amadori reactions producing AGEs. There has been increasing interest in the role of oxidation in the reactions between reducing sugars and proteins. The term glycoxidation is now used to define reactions that involve both reaction with a sugar and an oxidative step.

Apart from two reactions, the reversal of the Amadori reaction with the regeneration of Schiff's base and possibly the formation of 3-deoxyglucosone, all reactions of the Amadori product documented so far require aerobic conditions. These reactions include the formation of CML [Ahmed et al, 1988], pentosidine [Grandhee and Monnier, 1991], sugar-induced fluorescence and crosslinking [Fu et al, 1992] and other products of the 'browning reaction' [Fu et al, 1992]. Pyrraline and AFPGs are believed to be formed through interactions between 3-deoxyglucosone and free amino groups on proteins, although it is not clear yet if an oxidation step is involved in this reaction [Hayase et al, 1989].

All these 'glycoxidative' processes must be transition metal catalysed, as dioxygen exists in the triplet ground state and biomolecules such as proteins exist in singlet ground state. This makes reaction between them a spin forbidden process. Although the reaction is thermodynamically favourable, differences in the spin multiplicity of reactants cause the activation energy of the reaction to be prohibitively high. Some transition metals can exist in several spin states and are usually co-ordinated to biomolecules by their 'd' orbitals. The association of O₂ to transition metals can also occur through the 'd' orbitals of the metal. This allows the transition metal to act as a bridge across the spin forbidden gap, and hence the activation energy of the reaction between O₂ and biomolecules is lowered sufficiently for it to proceed [Miller et al, 1990].

Experiments quoted throughout the literature show that these glycoxidative reactions are metal-catalysed oxidation reactions. Adding DTPA (a metal chelator) to in vitro glycation incubations prevents the formation of fluorescence and crosslinks in collagen and albumin [Fu et al, 1992; Wolff and Dean, 1987]. The inhibition of these reactions by metal chelators reinforces the idea put forward by Miller et al (1990) that traces of transition metals found in buffers such as phosphate and Tris are responsible for catalysing oxidation reactions.

1.6 The role of glucose autoxidation products

An alternative approach to the established glycation theory was put forward by Wolff and his group in 1987. This is not widely accepted [Harding and Beswick, 1988] and there is now a large amount of evidence that shows that this pathway can only be a very minor one (see Section 1.7). Wolff pointed out that glucose is an α -hydroxyaldehyde which is in equilibrium with its enediol. The enediol can undergo enediol oxidation in the presence of oxygen and a transition metal catalyst, forming an α -ketoaldehyde. This reaction occurs via the enediol radical anion and generates hydrogen peroxide. The α -ketoaldehyde is postulated by Wolff to react with a free amino group on protein to give a ketoaminemethylol which rearranges to a ketoimine (Figure 1.3). The ketoimine would be the first stable protein-sugar adduct and it would be similar to the Amadori product (a ketoamine). Unfortunately Wolff's theory has been difficult to test because the product of the ketoimine in the sodium borohydride glycation assay that he used is indistinguishable from the product formed by the ketoamine (or Amadori product).

Wolff and Dean (1987) showed by incubating BSA and glucose in the presence and absence of the transition metal chelator DTPA, that 45% of protein-sugar adducts were formed by the glucose autoxidation pathway. More recent work from Baynes' laboratory shows that the formation of Amadori compounds is independent of whether the reaction takes place under aerobic or anaerobic conditions, although post-Amadori compounds are only produced under oxidative conditions [Fu et al, 1992]. This implies that it is the Amadori product rather than the ketoimine that is the major product when glucose and proteins are incubated under either oxidative or non-oxidative conditions. Therefore it is unlikely that the autoxidation of glucose is a major reaction pathway through which AGEs are formed.

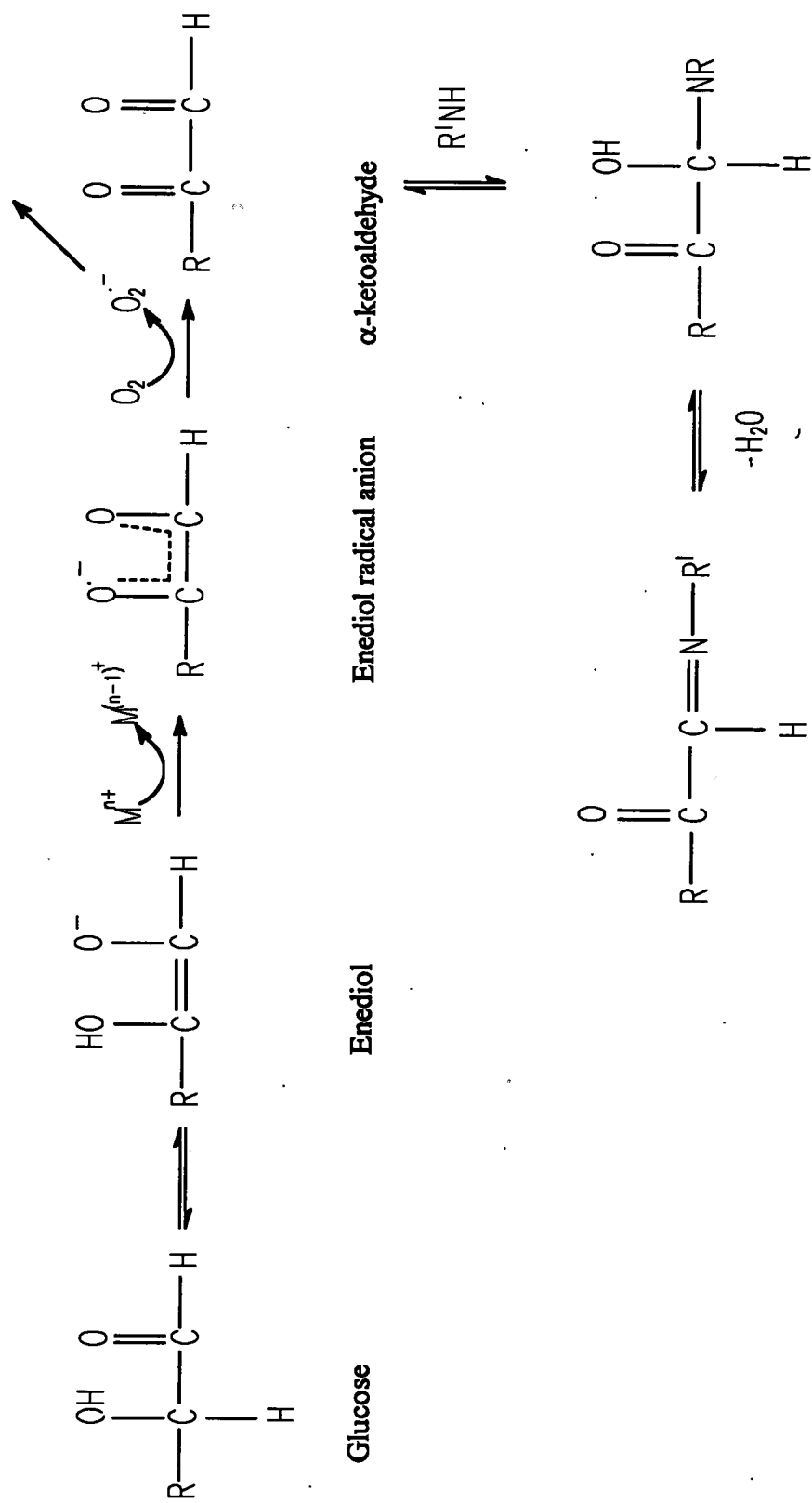


Figure 1.3: Autoxidative Glycosylation

1.7 Radical reactions and the Amadori product

There is increasing evidence that AGE formation involves post-Amadori reactions in which free radicals are generated by the autoxidation of Amadori product. Gillery et al (1988) detected the formation of superoxide from model Amadori compounds and from glycated proteins, but not from glucose, suggesting again that the autoxidation of glucose under physiological conditions is not a major reaction pathway. Superoxide radical production from model Amadori products, i.e. from incubations of reducing sugars and amino acids, was also shown by Azevedo et al (1988) by following the reduction of NBT. Kawakishi et al (1991) also found that the Amadori product mediated protein fragmentation similar to that caused by oxygen radicals, and suggested that the Amadori product could undergo autoxidation reactions similar to the one outlined in Figure 1.3. It seems that the most likely route for autoxidative damage would be via the Amadori product, as work carried out in the three groups mentioned above shows that the Amadori products produced oxygen radical species, whilst none were detectable in incubations of reducing sugars alone.

Glycated proteins have been found to act as both radical scavengers and producers of superoxide radicals. Glycated BSA scavenges both hydroxyl radicals and hydrogen peroxides to a greater extent than non-glycated BSA, although the glycated BSA has a lower scavenging ability for superoxides than the non-glycated BSA [Okamoto et al, 1992]. Hence with some glycated proteins the role of free radicals may be difficult to assess, particularly as it has been postulated by Halliwell (1988) that radicals once produced, immediately attack the protein and are not released into solution.

The production of free radicals by the oxidation of glycated proteins in the presence of catalytic transition metals has been shown to enhance lipid peroxidation in membranes and linoleic/arachidonic acid vesicles [Mullarkey et al, 1990]. Lipid peroxidation has been initiated by a glycated polylysine molecule that is associated with an iron^{III} ion [Sakurai et al, 1990].

1.8 Protein fragmentation

The non-enzymatic fragmentation of three different proteins (BSA, crystallin and LDL) has been shown to occur by incubation of protein with reducing sugars [Hunt et al, 1988a; Hunt and Wolff, 1990; Hunt et al, 1990]. In the absence of sugar fragmentation can be induced by radiolysis of the protein [Wolff and Dean, 1986; Davies, 1987] and by incubation of the protein with radical generating systems such as ascorbate/ Cu^{II} [Marx and Chevion, 1985]. It was also found that BSA could be fragmented by incubating it with model Amadori products in the presence of Cu^{II} [Kawakishi et al, 1990]. In every fragmentation system except direct radiolysis, the presence of Cu^{II} ions was required for the reaction to proceed, and it was inhibited by the inclusion of metal chelators such as DTPA and EDTA [Fong et al, 1987; Hunt et al, 1988a; Kawakishi et al, 1990].

Many experiments have been carried out to demonstrate the free radical nature of the fragmentation reaction, and to attempt to identify the reactive species. Extensive studies by Davies and Deloignore (1987) using ^{60}Co irradiation have shown that the hydroxyl radical initiates reactions that alter the structure of BSA, but in order for fragmentation to be observed both molecular oxygen and the superoxide radical must be present. Inhibition of fragmentation was observed on addition of the hydroxyl radical scavengers benzoic acid, deoxyribose and sorbinil to the glucose fragmentation system of Hunt et al (1988a) and on addition of mannitol to the ^{60}Co fragmentation system of Davies and Deloignore (1987). This shows that the hydroxyl radical is an active species in the fragmentation of proteins whether the reaction is initiated by irradiation or a glucose/ Cu^{II} system.

Fragmentation of BSA incubated with Amadori product or glucose is inhibited when catalase is included in the incubations, showing that hydrogen peroxide is necessary for protein fragmentation under these conditions [Kawakishi et al, 1990; Hunt et al, 1988a]. Direct incubation of BSA with H_2O_2 and either Cu^{II} or Fe^{III} has also produced protein fragmentation [Wolff and Dean, 1986]. It is likely that the hydrogen peroxide undergoes the metal- catalysed

Fenton reaction and produces hydroxyl radicals if the mechanisms of protein fragmentation deduced from the radiolysis work [Davies and Deloignore, 1987] (where hydroxyl radicals have been found to be the initial reactive species) apply in this reaction.

Hunt et al (1988a) put forward the idea that protein fragmentation was not dependent on the production of Amadori product, but was a result of the glucose autoxidation reaction.

However, the fragmentation of BSA incubated with Amadori product in the presence of Cu^{II} shows that Amadori products do play a major role in the fragmentation of proteins [Kawakishi et al, 1990], and this is further evidence against glucose autoxidation being a major pathway for "glycoxidative damage".

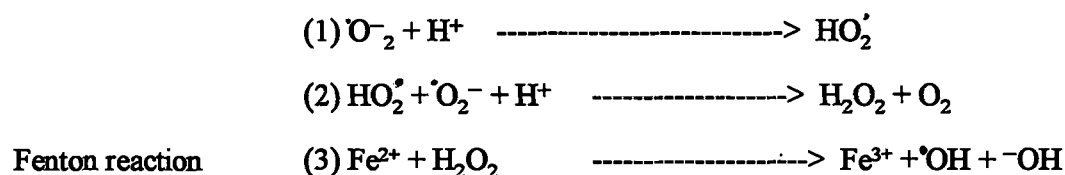
These reports suggest that sugar-induced protein fragmentation is one of the major post-Amadori reactions and may therefore be responsible for diabetic complications. The mechanism of sugar-induced fragmentation was therefore investigated in this study.

1.9 Lipid Peroxidation

The peroxidation of lipids is of interest in this study because serum albumin is a lipid transporting protein. *In vivo*, HSA has 1-2 moles of fatty acid bound per mole of protein. It has been reported that the peroxidation of lipids produces fluorescence with maxima similar to those of the products of glycoxidative reactions [Jones and Lunec, 1987]. In purified fraction V HSA, approximately 65% of bound fatty acids are unsaturated with about 6% of the total being polyunsaturated (containing three or more olefinic bonds) [Saifer and Goldman, 1961]. It is important to note that saturated fatty acids do not undergo lipid peroxidation reactions and mono- and di-unsaturated fatty acids do so only at very slow rates [Halliwell and Gutteridge, 1989].

Lipid peroxidation is initiated by the abstraction of a proton from a methylene group. This is facilitated if the adjacent carbon atom is a olefinic carbon, as the methylene C-H bond is weakened by the close proximity of the double bond. The abstraction of the proton leaves a carbon-centred radical. This undergoes a molecular rearrangement in which the carbon centred radical is stabilised by the production of conjugated double bonds (conjugated dienes absorb at 234nm and this is often used as a measure of the extent of lipid peroxidation). The carbon centred radical can combine with oxygen to form a peroxy radical, form crosslinks with another radical, or attack a protein molecule. This reaction can be propagated by peroxy radicals as they are capable of abstracting protons from methylene groups [Halliwell and Gutteridge, 1989].

The abstraction of the methylene proton that initiates lipid peroxidation can be by a hydroxyl or peroxy radical. The superoxide radical is insufficiently reactive to abstract a proton from lipids, although if Fe^{2+} is present then hydroxyl radicals can be produced through the scheme shown below.



Superoxide has been shown to form from Amadori products [Gillery et al, 1988; Kawakishi et al, 1991; Azevedo et al, 1988]. Therefore, if the superoxide radical is capable of forming a hydroxyl radical in the presence of Fe^{2+} under conditions that favour the Fenton reaction, then it might be expected that glycated proteins would be able to act as initiators of lipid peroxidation.

A study by Hicks et al (1988) showed that both glycated collagen and glucose are capable of catalysing lipid peroxidation in linoleic/arachidonic acid vesicles. The lag phase before hydroperoxide production was dependent on either the amount of glucose present, or the extent of glycation of the collagen, whereas the final rate of lipid peroxidation was independent of this.

Malondialdehyde is one of the many secondary products of the lipid peroxidation reaction and is formed from cleavage of the carbon backbone of alkoxyl radicals. It is an extremely reactive dicarbonyl compound, which when incubated with proteins *in vitro* forms fluorophores and crosslinks. The fluorophores are similar to those found in aged tissues with excitation maxima at 350-400nm and emission maxima at 450-470nm. Fluorophores derived from the reactions of linoleic, linolenic and arachidonic acids with proteins have excitation and emission maxima in the range of those of glucose-derived fluorophores [Kikugawa and Beppu, 1987].

1.10 Methods for measuring glycation

Although this thesis is mainly concerned with post Amadori reactions, glycation has been measured in some cases in order to determine which part of the reaction sequence is affected by factors such as lipid or alternative reducing sugars. There is a wide variety of techniques used throughout the literature and differences in the techniques should be taken into account when comparing results.

Two main classes of techniques are used to determine the extent of glycation of proteins. The first depends on the separation of glycated and non-glycated proteins and the subsequent determination of the amount of protein in each fraction. Techniques relying on the separation of glycated from non-glycated proteins include isoelectric focusing, gel electrophoresis, ion-exchange chromatography and affinity chromatography using phenyl boronate affinity gel. Of these methods, phenyl boronate affinity chromatography is the most commonly used, but results from this are not consistent between labs as it varies widely with chromatographic conditions. However, the use of an automated form of this assay is used throughout clinical labs. With all separation techniques each protein molecule can only be counted once, so even if a protein molecule has more than one mole of Amadori product bound to it the results from these techniques will read it as one, and hence give an underestimation of the true value of the extent of glycation [Furth, 1988].

Chemical assays ensure that every mole of Amadori product is measured, so even on multi-glycated proteins they probably give a more accurate value for the total glycation of proteins. There are four principal chemical assays used to measure glycation.

1.10.1 Serum fructosamine assay

This assay utilises the reducing power of the enediol, that forms on alkali treatment of the Amadori product. The amount of enediol produced is quantified by the amount of blue colour formed as nitroblue tetrazolium is reduced. There is no fixed endpoint to this assay and standardising it can be difficult, but for clinical uses this assay has become automated to help reduce problems associated with the standardisation.

1.10.2 Borohydride reduction

Borohydride reduces the specific double bonds ($C=N$ in the Schiff's base and $C=O$ in the Amadori product) to a stable sugar-amino acid link which is able to withstand boiling in 6M HCl. Boiling in HCl hydrolyses the peptide bonds and the sugar-amino acid adducts are then quantified by amino acid analysis. This can be one of the most sensitive of the glycation assays if radio labelled reagents are used.

1.10.3 TBA assay

This assay is one of the oldest chemical assays used for glycation. The glycated proteins are boiled in oxalic acid, releasing 5-hydroxymethyl furfural (HMF). The HMF is colorimetrically quantified by reacting it with TBA after the protein has been removed from solution by acid precipitation. This assay is not specific to Amadori product and any hexose will give HMF on boiling with oxalic acid, although the Schiff's base does not interfere with this assay.

1.10.4 Periodate oxidation

The periodate reagent cleaves molecules between adjacent hydroxy groups or, as occurs in Amadori products, between hydroxy and carbonyl groups. The major product from this reaction is performic acid, but one of the minor products, formaldehyde, is released only from C-1 and C-6 in straight chain hexoses that are adjacent to a carbon atom with either a hydroxy or a carbonyl group. This property makes the measurement of formaldehyde production (either by fluorescence after conversion to diacetyldihydrolutidine (DDL) or by absorbance of DDL at 412nm) [Ahmed and Furth, 1991] a particularly specific assay of Amadori product. It is a version of this assay adapted for a microplate reader that is used in this thesis.

1.10.5 Furosine assay

Although not strictly a chemical method, the furosine assay is probably the most sensitive and specific of the glycation assays, detecting only glycation of lysine residues. Glycation of the N-terminal residues must be assayed by amino acid analysis or one of the other methods mentioned above. Glycated proteins that have not been pre-treated in any way are hydrolysed by boiling in 6M HCl, producing furosine. The furosine is separated from the other products of acid hydrolysis by HPLC and detected by absorbance at 280nm. There is only a 30% yield of furosine from lysine-bound Amadori product, but the assay is not affected by Schiff's base, enzymatically bound sugar residues or free sugar.

1.11 Human Serum Albumin

The protein used in much of the work in this project was human serum albumin (HSA). HSA is a nonglycoprotein which makes up approximately 60% of plasma proteins. It is a single chain polypeptide of 585 residues and has a molecular mass of 66.4kD. It contains only one tryptophan residue [Peters, 1985]. It has a high number of charged residues; 36 aspartic acid, 62 glutamic acid, 59 lysine and 24 arginine and has a net charge of -15 at pH7. It is this high concentration of charged residues that helps to make HSA a soluble protein. There are 35 cysteine residues in HSA forming 17 disulphide bridges which hold the molecule in 9 loops and contribute to its high stability [Peters, 1985]. The 9 loops form three domains which have a large amount of amino acid homology, and are each further divided into two subdomains, A and B (Figure 1.4). Subdomains IA, IB and IIA are closely packed together forming the compact head of the molecule, whilst subdomains IIB, IIIA and IIIB are more loosely associated and form the elongated tail of the molecule [Carter et al, 1989]. The overall shape of the HSA molecule is an ellipsoid and its dimensions are 40 x 140Å with an axial ratio of 3.5. Not all the molecular space is taken up by the polypeptide chain, and approximately 20 water molecules are associated with specific sites on HSA. In addition there is the hydration shell of water molecules that are hydrogen-bonded both to the protein and to one another [Peters, 1985].

HSA functions as a transport protein and is able to bind a large range of ligands including inorganic cations, organic anions, various drugs, amino acids, bilirubin hemin and fatty acids. Most associations with HSA involve hydrophobic interactions, although a few classes of compound bind to HSA either by ionic interactions or covalently. Possibly the most important hydrophobic interactions *in vivo* are between HSA and long chain fatty acids.

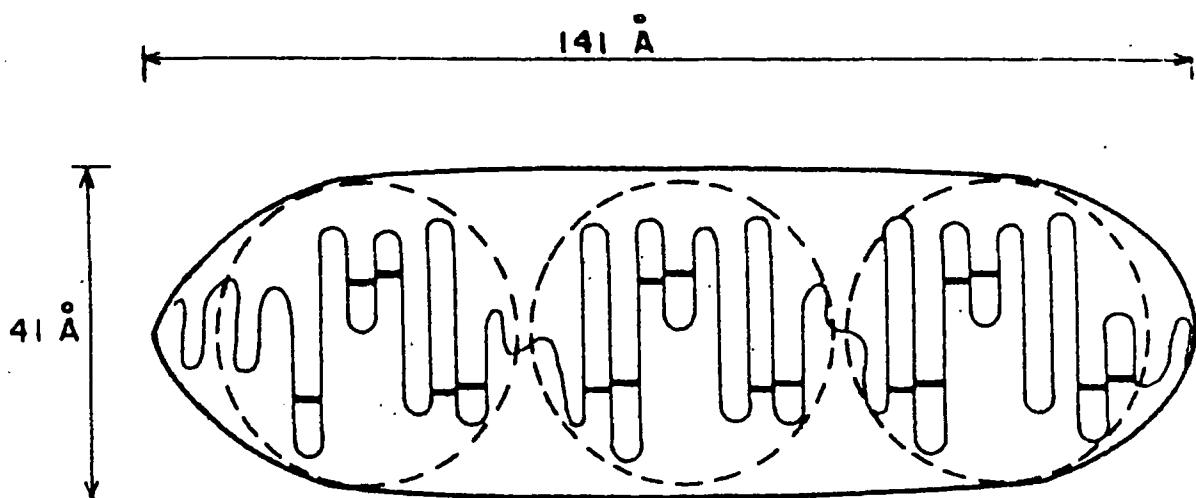


Figure 1.4: Model of serum albumin molecule [Peters, 1985]

1.11.1 Binding of long chain fatty acids to HSA

The binding of long chain fatty acids to HSA is of particular interest to this project because of the possibility of them undergoing peroxidation reactions, and because of the conformational change they cause in HSA when they bind.

The maximum number of fatty acids that can be loaded onto HSA is 6, both *in vivo* and *in vitro*, although *in vivo* a loading of 6 is normally only found after renal dialysis and even after severe exercise the maximum loading is 4. *In vivo* HSA usually carries 1-2 moles of fatty acid per mole of protein and these can be removed from the protein without denaturing it by using charcoal treatment [Peters, 1985; Chen, 1967].

The binding constant for the binding of fatty acids to HSA is about two orders of magnitude higher than for most other ligands that bind to HSA, with the main energy of binding being due to hydrophobic interactions [Brown and Shockley, 1982]. The long chain fatty acids bind in their salt form to HSA. The central part of the fatty acid chain is held rigidly, whilst the ends are free to move. The binding affinity of fatty acids increases with chain length for the first 2-3 high-affinity binding sites, indicating that hydrophobic interaction rather than ionic interaction is the major binding force [Peters, 1985].

The fatty acids bind to HSA with graded affinity, the fourth fatty acid to bind having less than one tenth of the binding affinity of the first [Peters, 1985]. The strongest binding site has been shown to be subdomain IIIA. The remaining 3 specific sites of fatty acid binding, in order of affinity, are the interface between domains II and III, the centre of domain I and the centre of domain II [Carter et al, 1989; Peters, 1985]. The binding of fatty acids at the first two binding sites (subdomain IIIA and between domains II and III) causes a conformational change in the molecule in which the C-terminal end (subdomains IIIA, IIIB and IIB) is compacted. This results in a change in the dimensions of the molecule to 40 x 130 Å with the axial ratio changing from 3.5 to 2.2 [Dröge et al, 1988; Peters 1985].

1.11.2 Binding of tryptophan, tyrosine and organic anions such as drugs

The amino acids tryptophan and tyrosine both bind to HSA at the same site and are the only amino acids to bind to HSA non-covalently. Competition data shows that the same site also binds short chain fatty acids and organic anions such as diazephines, naproxen, clofibrate and flufenamic acid. The binding site has been termed as 'site II' and is located in domain III. The binding affinities for the organic anions are in the range of $\log K_A = 4$ to 6 whereas for long fatty acids $\log K_A$ is about 8. The binding of ligands at this site is affected if more than 2-3 moles of long chain fatty acids are bound to HSA. It is not known whether this is because of the conformational change induced by the binding of the long chain fatty acids or because the long chain fatty acids bind at this site themselves [Peters, 1985].

1.11.3 Ligands that bind covalently to HSA

The covalent binding of glucose and other reducing sugars to HSA has been reported widely and is of particular interest in this study (see Section 1.12). Several other classes of compound bind covalently to HSA. They include pyridoxal phosphate, aspirin and amino acids which form mixed disulphide bonds with residue Cys-34. The covalent attachment of aspirin is a result of a condensation reaction between its acetyl group and the ϵ -amino group on residue Lys-199, which is one of the major sites of *in vitro* glycation.

1.11.4 Binding of inorganic cations

i) Calcium

Half the plasma calcium is bound to HSA and an average of two moles of Ca^{2+} per mole of HSA is normally found *in vivo*. The binding of calcium to HSA is not specific and the binding constant is low [Peters, 1985].

ii) Copper

As AGEs are thought to form via metal-catalysed oxidation reactions, the binding of copper to HSA may be important in the formation of its glycoxidative products. Albumin binds approximately 7% of serum copper. The remaining copper is tightly bound to ceruloplasmin and is not exchangeable *in vivo*. The copper bound to albumin is rapidly exchanged with the copper in the tissues and therefore albumin is considered to be a major transport protein of copper [Sarkar, 1983]. The copper bound to ceruloplasmin is not oxidatively active but copper bound either to albumin or His residues is still able to catalyse the Fenton reaction (see Section 1.9), although the reactive OH radical species formed is thought to attack the molecule to which the copper is bound, and the radical is not released into solution [Halliwell and Gutteridge, 1989].

Under normal conditions there is less than one mole of copper per mole of albumin, but in conditions such as Wilson's Disease (a hereditary deficiency of ceruloplasmin) copper levels in serum are elevated. Although early work on albumins showed that they had many copper binding sites, the presence *in vivo* of less than one equivalent of copper makes the first copper binding site of much greater physiological importance than the other sites. Early work carried out by Naik et al (1975) on BSA, showed two distinct classes of binding site. The first three sites had a high affinity for copper ions, whilst the remaining 16 showed a much weaker affinity.

The average binding constant for the three high affinity sites is $\log K_A=6.5$, and the binding constant for the remaining sites is $\log K_A=4.3$. These studies were carried out using a cupric-ion-specific electrode [Naik et al, 1975]. Later work has shown that most albumins have a single high affinity copper binding site, with further lower affinity binding sites.

Not all serum albumins bind Cu^{II} with the same affinity at a single high affinity binding site. It has been shown that BSA, HSA, and rat serum albumin (RSA) had a preferential binding site of high affinity for one Cu^{II} ion. This binding site is not present in chicken serum albumin (CSA) or dog serum albumin (DSA) which led to further study of the N-terminal binding site [Appleton and Sarkar, 1971]. Before the N-terminal sequences of these albumins had been completed it was established that the His residue which forms part of this binding site is absent in DSA.

In a recent paper by Predkl et al (1992), this high affinity binding site was characterised and compared to the N-terminal sequences of RSA, HSA, BSA, porcine serum albumin (PSA), DSA, and CSA. The N-terminal co-ordination site is square planar using the N atom of the His residue in position 3 of the amino acid sequence. In DSA and PSA there has been a substitution to a Tyr residue and in CSA an insertion of Glu at position 3 puts the His residue into position 4. DSA binds the first equivalent of copper at two sites of equal specificity, although these sites are of much lower affinity than the high affinity site of HSA [Appleton and Sarkar, 1971]. Predkl et al found that co-ordination of metals to DSA and CSA are similar, showing that the His residue has to be at position 3 if the binding site is to have a high affinity.

The postulated high affinity site on BSA and HSA is square planar with the four co-ordinating atoms being the N-terminal N atom, 2 backbone N atoms and the imidazole N atom from the His residue in position 3. The aspartyl carboxylate group from position 1 in HSA also co-ordinates to the bound Cu^{II} ion. In CSA and

DSA, the square planar co-ordination site has the N-terminal N atom and three backbone N atoms as the "corners" [Laussac and Sarkar, 1984]. Synthetic polypeptides to mimic the square planar binding site of albumins have been made by the laboratories of Sarkar [Laussac and Sarkar, 1984]. Both the peptides prepared had a His residue in position 3 and have been found to bind Cu^{II} ions with similar affinity to the principal copper binding albumins.

iii) Nickel

Nickel binds to HSA at the same site as copper, but with a much lower affinity. The binding constant for nickel is $\log K_A=9.6$ and the binding constant for copper binding at this site is $\log K_A=16$. As a result of this difference in binding affinities, nickel is displaced from the specific binding site on HSA by copper [Peters, 1985].

iv) Other metals

The binding of metals other than copper and nickel seems to be much more ionic in nature. The association constants for Zn^{II} and Mn^{II} are 6 and 5 orders of magnitude less than for Cu^{II} respectively. Despite this relatively low binding affinity, two thirds of the plasma Zn^{II} not already bound to α_2 -macroglobulin is found bound to HSA. Mercury binds to the thiol group of residue Cys-34 and Sarkar et al (1983) found that a dimer of albumin was formed through binding to one Hg^{II} ion. Gold also binds to HSA with some sort of association with the thiol group of Cys-34.

1.12 Glycation of HSA

1.12.1 Sites of glycation of HSA

In vivo the principal site of glycation is Lys-525, which is in domain III. Nearly half of all *in vivo* glycation occurs at this residue. This was determined by treating *in vivo* glycated HSA with NaB^3H_4 followed by tryptic digestion. The glycated fragments were then adsorbed to an Affi-Gel 601 boronic acid affinity column and the glycated residue Lys-525 was identified. This lysine residue is the second or carboxyl lysine of a lysine-lysine sequence [Garlick and Mazer, 1983]. The first lysine is thought to catalyse the rearrangement of the Schiff's base to the Amadori product by acid catalysis. At least a further 8 of the 59 lysine residues in HSA are glycated *in vivo*. The three lysine residues which are most often glycated after Lys-525 are Lys-439, Lys-281, and Lys-199. Lys-233, Lys-317, Lys-12 and Lys-534 are also glycated *in vivo*, but to a lesser extent [Iberg and Flückiger, 1986].

In vitro, residue Lys-199 is the principal site of glycation. This residue is on the interdomain peptide between domains I and II [Carter et al, 1989]. Glycation starts with nucleophilic attack by an unprotonated amine group on a glucose (or other reducing sugar) in its straight chain form. The product of this reaction is a Schiff's base. At physiological pH very few primary amino groups are unprotonated. The pK of Lys-199 is thought to be 8.0-8.7 whereas other lysine residues in HSA have much higher pK values which are in the region of 11. Lower pK values of lysine residues favour both the formation of the Schiff's base and its subsequent rearrangement to the Amadori product, as both of these reactions can only proceed when the amino group is uncharged [Iberg and Flückiger, 1986; Garlick and Mazer, 1983].

The pK of the amino group is not the only factor which determines which lysine residue is glycated, because despite having a more unfavourable pK value than Lys-199, Lys-525 is glycated in preference to Lys-199 *in vivo*. A possible explanation for this is that Lys-199 is

sterically hindered by fatty acids or other large molecules which would prevent the sugar molecule from reaching the amino group. These large molecules may be inadvertently removed from the HSA during the purification process which precedes any *in vitro* work [Garlick and Mazer, 1983]. Murtiashaw and Winterhalter (1986) showed that glycation of albumin up to 3 moles of glucose per mole of protein did not affect the binding of palmitate to HSA, even though the residue Lys-525 has been implicated as being in the primary fatty acid binding site

The formation of Schiff's base is a reversible process, so if conditions are unfavourable for the Amadori rearrangement to proceed, then much of the Schiff's base formed at that residue will dissociate before the reaction sequence can continue. This could explain why the choice of glycation site is not solely dependent on the pK of the lysine residue. The Amadori product is much more stable than the Schiff's base, but the Amadori rearrangement is also a reversible process which can be accelerated by acid-base catalysis (see Section 1.2).

Glucose uptake *in vitro* is fastest in the first two days of incubation, after which the rate of glucose incorporation decreases. This could be because most readily glycosylated sites becoming used up in the first two days [Mereish et al, 1982]. Mereish also found that the rate and extent of glycation of HSA are both increased when fatty acid molecules are removed from HSA, and that the initial rate of glycation is much higher in delipidated HSA than in native HSA. However, the delipidated HSA used by Mereish was prepared by heating at very low pH which may have caused the hydrolysis of Amadori products already on the protein. The total amount of Amadori product present on each of the forms of HSA was not recorded, because he measured the uptake of radioactive glucose. HSA is not denatured by the charcoal method of delipidation [Chen, 1967], and it was this preparation which was used in my work.

It has been shown that the main *in vitro* site of glycation is Lys-199, instead of Lys-525 which is the main site of glycation *in vivo*. Lys-199 is also the binding site for salicylate and it has been observed that the glycation of this residue interferes with the binding of

salicylate. In binding studies it has been shown that glycated HSA has only one class of salicylate binding site, whereas partially and non glycated HSA have two classes of binding site for salicylate [Mereish et al, 1982]. It has also been suggested that up to 25-30% glycation of HSA there is no appreciable decrease in salicylate binding to HSA. The accepted figure for glycation of HSA in diabetics is 25-30%, so it is unlikely that there is any impairment of salicylate binding *in vivo* [Mereish et al, 1982].

1.12.2 Extent of glycation of *in vivo* HSA

The extent of glycation of HSA in normal individuals is between 10% and 12% when phenyl boronic acid affinity gel is used to separate glycated from non-glycated HSA. However, it has been shown that HSA glycated at the ϵ -amino lysine residue is not completely retained. The proportion of glycated HSA bound to the phenyl boronic acid affinity resin varies with the chromatographic conditions, and the normal values for the percentage of HSA molecules reflect this (estimates vary between 6.3% and 15.5%) [Schleicher and Wieland, 1989]. When HPLC is used the normal value for percentage of HSA glycated is 20% [Schleicher and Wieland, 1989]. This value of 20% is still low compared to the 25% which is found when chemical assays are used [Baynes et al, 1984]. One explanation for this discrepancy could be that some molecules will be multi-glycated (i.e. have more than one bound sugar residue) and the chromatography methods will count the protein molecule only once, whereas the chemical methods will measure each sugar molecule which is covalently bound.

The value of 25% glycation of HSA *in vivo* is much higher than the *in vivo* value for haemoglobin which is 7.5% [Olufemi et al, 1987]. Unlike haemoglobin, which is principally glycated at the N-terminal valine of the β -chain (alpha glycation), albumin is glycated at the ϵ -amino groups of certain lysine residues, and little alpha glycation has been shown to occur, although Olufemi et al (1987) suggest that a small amount of HSA glycation does occur at the N-terminal.

1.12.3 Recombinant HSA

A sample of recombinant HSA was provided by Delta Biotechnology Ltd., enabling me to carry out investigations on samples of HSA that had well defined lipid content.

Recombinant HSA has been synthesised in brewers yeast (by gene insertion) and extracted and renatured. The properties of this protein are very similar to those of native HSA, although there are slight differences in bilirubin binding curves, and spectra have shown that some uv absorbing non-protein impurities are present in recombinant HSA. The metal chelator EDTA is included in the buffers at every step of the extraction and purification processes, so recombinant HSA has a very low Cu^{II} content [Quirk et al, 1989]. The lipids that are bound to recombinant HSA are 97.6% saturated and 2.4% unsaturated, with only 0.01% of the total fatty acids being polyunsaturated [Delta data sheet].

1.13 Transferrin

Transferrin was used in some of these studies because it is an iron binding protein which complements the use of the copper binding protein HSA. Human transferrin is a single chain polypeptide of 678 amino acid residues and a molecular weight of 79,550 daltons. This includes the two Asn linked glycan moieties each of molecular weight 2207 [MacGillivray et al, 1982]. Transferrin reversibly and strongly binds two iron atoms per molecule. Transferrin functions mainly as an iron-transport protein, although it is also believed to play a role in protection against micro-organisms by denying them the iron they need to grow. It is found mainly in the extracellular fluid and the serum. Its serum concentration is 2-4 mg/ml and it is usually about 30% iron saturated *in vivo* [Brock, 1985]. Iron is not an inherent part of transferrin so the protein can fold correctly in the absence of iron [Bailey et al, 1988].

The transferrin molecule is ellipsoidal in shape with the ratio of major to minor axes 3:1 in the case of the apoprotein, and 2.5:1 when the protein has two iron ions bound. The polypeptide chain is folded into two lobes which are held by 19 disulphide bridges, 8 of which are in the N-terminal lobe and 11 in the C-terminal lobe [Bailey et al, 1988; Brock, 1985]. Both the glycan moieties are on the less flexible C-terminal lobe, and are joined through β -N-glycosidic linkages to asparagine residues 415 and 608 [Aisen and Listowsky, 1980]. The two lobes have a large amount of amino acid homology, and when their sequences are compared it is found that 40% of the residues are identical, whilst many of the non-identical amino acid residues have been replaced by amino acid residues with similar chemical properties [MacGillivray et al, 1982]. A short polypeptide chain joins the two lobes and is a maximum of 7 residues long, as it falls between the half-cystine residues at the end of each lobe [MacGillivray et al, 1982].

Each lobe consists of two dissimilar domains, with the iron-binding site for each lobe being at the domain interface. The binding and release of iron at the binding site involves movements of the domains which open and close the binding cleft [Baker and Lindley,

1992]. The binding of metal appears to stabilise the structure of the transferrin molecule as the metal-loaded protein is more resistant to thermal and proteolytic attack than the apoprotein [Aisen and Listowsky, 1980]. The binding sites for the iron ions are estimated as being 25-43 Å apart [Heubers and Finch, 1987]. The iron binding cleft in both the C-terminal and N-terminal lobes contains a lysine residue (Lys-206 in the N-terminal cleft and Lys-534 in the C-terminal cleft). These appear to play no part in the co-ordination of the iron ion to transferrin, although both undergo a large change in reactivity on the binding of iron to apotransferrin [Bailey et al, 1988].

In the absence of iron, transferrin will bind other metals *in vitro* including gallium, copper, chromium, cobalt, manganese, vanadium, aluminium, europium, plutonium and platinum, but all these metals are displaced from the specific binding clefts by iron. *In vitro* binding of these metals should not be taken as evidence of *in vivo* binding. *In vivo*, even though there are metal binding sites available on transferrin and serum copper levels are similar to serum iron levels, no appreciable amount of copper is found bound to transferrin. Although transferrin can bind Zn^{II} *in vitro*, it is unlikely to be bound to transferrin in serum, as HSA is present at high concentrations and binds Zn^{II} with a much greater affinity than transferrin [Heubers and Finch, 1987; Brock, 1985]. More than two equivalents of Cu^{II} and Zn^{II} can be bound to transferrin although only the first two ions to bind are bound with high affinity [Brock, 1985]. Human serum transferrin has 19 histidine residues which may play a role in the non-specific binding of these metal ions [Hudson et al, 1972].

Transferrin incubated with 20mM glucose for seven days forms 1.1 moles of Amadori product per mole of protein [Ney et al, 1985]. Glycation of transferrin did not affect the rate or extent of iron binding, therefore it seems unlikely that either Lys-206 or Lys-534 (the lysine residues found in each of the iron binding clefts) are involved in the formation of Amadori product [Ney et al, 1985; Bailey et al, 1988]. *In vivo*, transferrin has 1.32 moles of Amadori product per mole of protein compared to 3.44 moles of Amadori product per mole of HSA. The half-life of transferrin is 8.5 days as opposed to 19 days for HSA [Zoppi et al, 1987], which may explain its lower glycation *in vivo*.

1.14 Inhibitors of AGE formation

It has been shown by many groups that the addition of metal chelators to *in vitro* glycation media inhibits the production of fluorescent AGEs, glucose-derived crosslinks and glucose-induced protein fragmentation [Fu et al, 1992; Hunt et al, 1988a]. These compounds include DTPA [Wolff and Dean, 1987; Fu et al, 1992], EDTA [Wolff and Dean, 1986] and desferrioxamine [Le Guen et al, 1992]. It has been shown by Fu et al (1992) that the metal chelator DTPA inhibits the formation of AGEs, but does not affect the formation of the Amadori product. Amadori product formation is also unaffected by the absence of oxygen during the reaction, although AGE formation does not occur under anaerobic conditions. Clearly the use of metal chelators and anaerobic conditions are only of use for *in vitro* studies, and therefore other potential inhibitors of the Maillard reaction for use *in vivo* have been investigated. A brief outline of the four potential inhibitors used in this study is given below.

1.14.1 Aminoguanidine

Aminoguanidine is a nucleophilic hydrazine with three amino groups (Figure 1.5). It was first used in 1986 [Brownlee et al] when it was found to inhibit fluorescence but not the formation of Amadori products. It has been reported that aminoguanidine inhibits the covalent attachment of radio labelled sugars to lens proteins [Lewis and Harding, 1990]. Recent work by Requena et al (1993) has investigated more thoroughly the mechanism by which aminoguanidine inhibits AGE formation. Both the fructosamine and furosine assays were used to monitor Amadori product formation. Including aminoguanidine in the incubation medium did not reduce the amount of Amadori product formed. However, at high concentrations of aminoguanidine, the fructosamine assay gave a lower reading whilst the furosine assay gave a constant reading, which demonstrates blocking of Amadori groups by aminoguanidine (See Section 1.10). Inhibition of fluorescence formation occurs at low

concentrations of aminoguanidine, showing that it is not due to either aminoguanidine reacting directly with the glucose or the blocking of Amadori groups by aminoguanidine, as this is only seen at high concentrations of the inhibitor. This suggests that aminoguanidine acts on a post-Amadori intermediate of the Maillard reaction. This agrees with earlier work carried out by Oimomi and Igaki (1989) in which aminoguanidine inhibits the *in vitro* development of fluorescence and crosslinks in proteins incubated with the post-Amadori dicarbonyl intermediate 3-deoxyglucosone. It has been postulated that 3-deoxyglucosone plays a large role in the development of AGEs [Kato et al, 1989].

1.14.2 Propyl Gallate

Propyl gallate is a synthetic antioxidant which like trolox is based on an aromatic ring (Figure 1.5). It prevents the propagation of radical reactions by donating a proton to radicals such as the peroxy and alkoxy radicals, forming a radical stabilised by delocalisation around the aromatic ring. This new radical is not sufficiently reactive to abstract further protons to propagate the reaction. The action of propyl gallate may be two-fold as it is also capable of binding transition metal ions which may prevent them from catalysing oxidative reactions. It is a water-soluble compound which is often added to foodstuffs as an antioxidant [Halliwell and Gutteridge, 1989].

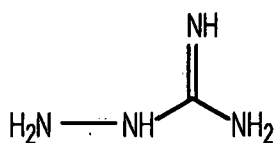
1.14.3 Trolox

Trolox is a water-soluble vitamin E derivative (Figure 1.5). Vitamin E is probably one of the major fat-soluble antioxidants found *in vivo*. It is a very effective inhibitor of lipid peroxidation reactions as it can donate a proton to peroxy or alkoxy radicals and prevent propagation of the reaction [Halliwell and Gutteridge, 1989]. Vitamin E reduces glycation of HSA as measured by phenyl boronic acid affinity chromatography in a dose-dependent

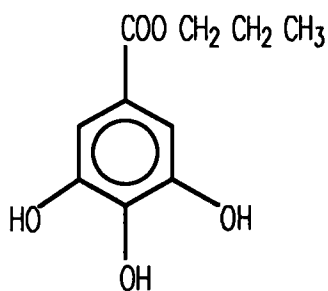
manner [Ceriello et al, 1988]. Vitamin E has also been shown to be an effective inhibitor of lipid peroxidation induced by a glycated polylysine-iron complex [Sakurai et al, 1991].

1.14.4 Acetyl Salicyclic Acid

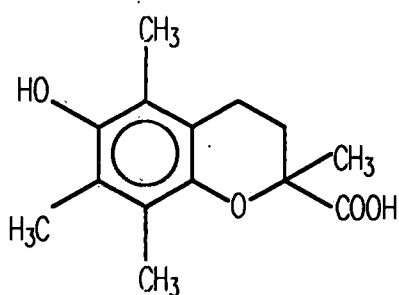
Acetyl salicyclic acid (or aspirin) is capable of acetylating proteins at the amino groups, thus blocking the formation of Amadori products. It was suggested by Cotlier and Sharma in 1981 from epidemiological studies, that acetyl salicyclic acid plays a protective role against the formation of diabetic cataracts. *In vitro* the inhibition of glycation corresponds to a reduction of aggregate formation in lens proteins, possibly because the acetylation of lens proteins causes less unfolding and aggregation of the proteins than glycation [Swamy and Abraham, 1989]. The structure of acetyl salicyclic acid is shown in Figure 1.5.



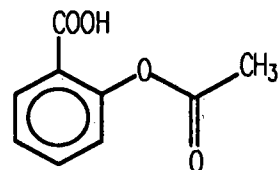
Aminoguanidine



Propyl Gallate



Trolox



Acetyl Salicylic Acid

Figure 1.5: Structures of aminoguanidine, propyl gallate, trolox and acetyl salicylic acid

1.15 Aims

In order for effective drug therapy for diabetic complications to be developed a better understanding of the reactions involved in the formation of AGEs is needed. Using fluorescence spectroscopy and gel electrophoresis the aims of this project were:

- i) to show that the changes observed in proteins incubated with sugars are in fact due to reaction of the protein with sugar rather than other reactions that occur with the ageing of proteins,**
- ii) to see the effect that the bound lipid on albumin has on the formation of Amadori product and on the post-Amadori reactions of glycofluorophore formation and protein fragmentation,**
- iii) to determine the role that transition metals play in the formation of glycofluorophores and the fragmentation of proteins, and to investigate whether the specific binding of transition metals to proteins influences these reactions,**
- iv) to elucidate the mechanism of these reactions by using inhibitors and to determine whether or not the products of glucose oxidation reactions are involved in the formation of AGEs.**

METHODS

2.1 *In vitro* incubations of proteins with glucose

Proteins were incubated at concentrations of 1, 10, 20 and 40 mg/ml with various concentrations of sugars (usually glucose). Most of the incubations were in sodium phosphate buffer of pH 7.4 although in certain experiments HEPES buffer (also pH 7.4) was used. Sodium azide (3mM) was included in the incubations to prevent bacterial contamination. All incubations were at 37°C. At the end of the incubation period, samples were frozen to stop the reaction from proceeding any further.

2.2 Measurement of the extent of glycation

Samples to be assayed for Amadori product were dialysed extensively against 0.05M sodium phosphate buffer, pH 7.4, using a membrane with a molecular weight cut-off of 12000-14000 daltons. This was done to remove free sugars which interfere with the periodate assay.

2.2.1 Periodate Assay

An adaptation of the microplate version [Ahmed and Furth, 1991] of the periodate assay devised by Gallop et al (1981) was used.

Duplicates of all samples and standards were run throughout this assay. 60µl of each sample was placed in a microcentrifuge tube and 30µl each of 0.1M HCl and 0.5M NaIO₄ were added to each tube. The tubes were vortexed and allowed to stand at room temperature for 30 minutes before being placed on ice for 10 minutes to stop the oxidation reaction. Pre-cooled 15%ZnSO₄ (30µl) and 0.7MNaOH (30µl) were added to each tube. The tubes were vortexed and then centrifuged immediately at 12000rpm for 15 minutes using a Heraeus Biofuge A. 100µl of the supernatant from each sample or standard were put into the wells of a microplate and 200µl of formaldehyde detection reagent was added to each well. The microplate was mixed using a Wellmixx 1, and then covered and incubated at 37°C for 1 hour. The plate was allowed to cool to room temperature for 10 minutes before the absorbance at 405nm was read using a Microplate reader (Bio-tek EL311). Formaldehyde detection reagent was prepared fresh every time by dissolving 2.54g of ammonium acetate and 46µl of acetylacetone in water with a final volume of 10ml. A full set of fructose standards were run with every set of samples as this assay is both temperature and time dependent.

2.2.2 Protein Determination

The concentration of protein in samples after dialysis had to be determined in order to calculate the number of moles of Amadori product formed per mole of protein. This was measured using the Pierce BCA protein assay reagent (Prod no. 23225). Copper binds to the peptide bonds of proteins and under alkaline conditions the peptide-bound copper(II) will react to form copper(I). It is the peptide-bound copper(I) that reacts with bicinchoninic acid to produce the intense purple colour. The protocol provided by the manufacturer of this kit was followed and the absorbance was measured at 570nm using the microplate reader. A standard curve was run every time as this assay is both temperature and time dependent.

2.3 Measurement of AGEs

Both glycofluorophore production and the glucose-induced fragmentation of proteins were measured as part of the project.

2.3.1 Measurement of fluorescence

During the course of this study, three different fluorimeters were used. Initial fluorescence readings were carried out using an Aminco Bowman Spectrofluorimeter (model J4-8960). In later work both the Perkin Elmer LS30 and LS50B were used.

(i) Using the Aminco Bowman Spectrofluorimeter

Samples were diluted to a concentration of 1 μ M protein with water and the fluorescence measured at excitation 325 and emission 375nm. Quinine sulphate standards in 0.1M H₂SO₄ read at excitation 250nm and emission 450nm were used to calibrate the fluorimeter on a daily basis. The emission slit was 1mm and the excitation slit was 2mm. All samples were read in duplicate and were expressed as relative fluorescence. Unfortunately the xenon lamp in the fluorimeter was ageing and the fluorescence readings of the quinine sulphate standard was decreasing in value. This made it impossible to directly compare values obtained in early experiments with later experiments, although within each section of this thesis the results are always directly comparable. The same trends are always seen in duplicate experiments even if the absolute values could not be superimposed on earlier work. When a suitable replacement lamp was found, the power supply was no longer delivering a smooth current. Despite the efforts of technical staff at the Unit it was not possible to correct this fault making it necessary to find alternative equipment to finish this work.

(ii) Perkin Elmer LS30

A Perkin Elmer LS30 was kindly lent to us by Perkin Elmer whilst the purchase of a new fluorimeter was being arranged. It was not as sensitive as the Aminco Bowman had been so a higher concentration of protein in each sample was used (0.5mg/ml or 7.2 μ M of HSA). Fluorescence readings were recorded at both excitation 325nm/emission 375nm and excitation 370nm/emission 440nm for duplicates of all samples. Quinine sulphate standards were used to calibrate the fluorimeter and unlike the Aminco Bowman these values remained steady from day to day for both standards and samples. All samples were measured in a flow cell as there is no facility to use cuvettes with this model.

(iii) Perkin Elmer LS50B

Samples were read at a concentration of 0.5mg/ml in this fluorimeter. Unlike the LS30 model either a cuvette or flow cell could be used. The LS50B also enabled me to scan fluorescence and take second derivatives of the spectra, which neither of the other two machines were capable of. The daily calibration of this machine with quinine sulphate showed that it held a constant value from week to week.

2.3.2 Detection of protein fragmentation

Vertical gel electrophoresis was performed according to the method of Laemmli (1970) using 10% gels. However, piperazine di-acrylamide (PDA - Bio rad cat no. 161-0202) was used in the place of bis-acrylamide in order to reduce the background in silver stained ^{*}gels. The gels were run at a constant voltage of 175V for approximately 1 hour in a Bio-rad mini-PROTEAN II apparatus. Gels were stained using the Bio-rad Silver stain Plus kit (Cat no. 161-0449).

Samples were boiled in 2x buffer for three minutes prior to loading onto the gel, and approximately 1-2 μ g of protein in a volume of 5 μ l was added to each well. The composition of the 2x buffer is 0.125M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10%

2-mercaptoethanol and 0.002% bromophenol blue. Molecular weight marker kits from Bio-rad and Sigma with molecular weight ranges of 14-97kD and 14.2-66kD were used. In addition to these molecular weight standards lysozyme was added to some of the protein samples, immediately prior to treatment with the 2x buffer, in order to provide an internal standard. A band of approximately 45kD was observed, which is due to the presence of ovalbumin in the lysozyme supplied by Sigma.

In some cases, e.g. Figure 3.1.4, fragmentation is seen as a loss of starting material and/or a non-discreet smear of lower molecular mass, whilst in other cases, e.g. Figure 3.4.6, a series of discreet lower molecular weight bands can be seen.

The exact composition of the gels is shown in Table 2.1.

Table 2.1: Composition of Gels

	Resolving Gel (10%)	Stacking Gel (4%)
Acrylamide/PDA Stock	3.33 ml	0.65 ml
Water	4.02 ml	3.05 ml
1.5M Tris-HCl pH 8.8	2.5 ml	0
0.5M Tris-HCl pH 6.8	0	2.5 ml
10% SDS	100 μ l	50 μ l
10% Ammonium persulphate	100 μ l	50 μ l
TMED	15 μ l	10 μ l

The acrylamide/PDA stock solution had 30% acrylamide and 2.67% PDA. The composition of the running buffer was 0.025M Tris, 1.92M glycine and 1% SDS adjusted to pH 8.3.

Gels were photographed using type 53 Polaroid film in a Polaroid camera (Model MP-4) fitted with a green filter (no. 58).

2.4 Source of proteins

Fraction V native and delipidated HSA were both obtained from Sigma Chemical Company Ltd. (prod no. A 1653 and A 1887). The delipidated HSA was prepared by the method of Chen (1967), which uses charcoal to remove the lipid without denaturing the protein. Both forms of HSA were only 96 to 99% pure according to the manufacturer's details, although no impurities showed when these proteins were stained with Coomassie Blue on SDS-PAGE. However, where silver staining is used with SDS-PAGE these impurities can be seen.

The recombinant HSA was a gift from Delta Biotechnology Ltd. and has low levels of bound copper and polyunsaturated lipids [Quirk et al, 1989].

Transferrin was obtained from Sigma Chemical Company Ltd. (prod no. T 4515) and by the manufacturers analysis was approximately 95% pure.

RESULTS

3.1 Glucose dependency of AGE formation

At the start of this project some experiments were carried out to determine whether fluorescent products similar to AGEs form on proteins without the presence of reducing sugar in the incubation media. Figures 3.1.1, 3.1.2 and 3.1.3 show that under the conditions used for the incubations, little or no fluorophore production at excitation 325nm and emission 375nm occurs unless glucose is present. Similar experiments were carried out to determine the role glucose plays in the fragmentation of proteins. Figure 3.1.4 shows that no fragmentation of protein occurs under the incubation conditions used unless glucose is present in the incubation. Lanes A and B contain native and delipidated HSA respectively, incubated in the absence of glucose and lanes C and D contain the same proteins incubated under identical conditions except for the inclusion of glucose. Fragmentation of the protein is only seen in lanes C and D.

The results in all figures referred to above, and in the sugar-free controls run alongside the other experiments throughout this project, show that under the conditions used, no fluorescence forms and no fragmentation occurs unless a reducing sugar such as glucose is present in the incubation. Therefore, it is possible to follow the formation of AGEs without interference from changes in the protein that occur in the absence of sugars.

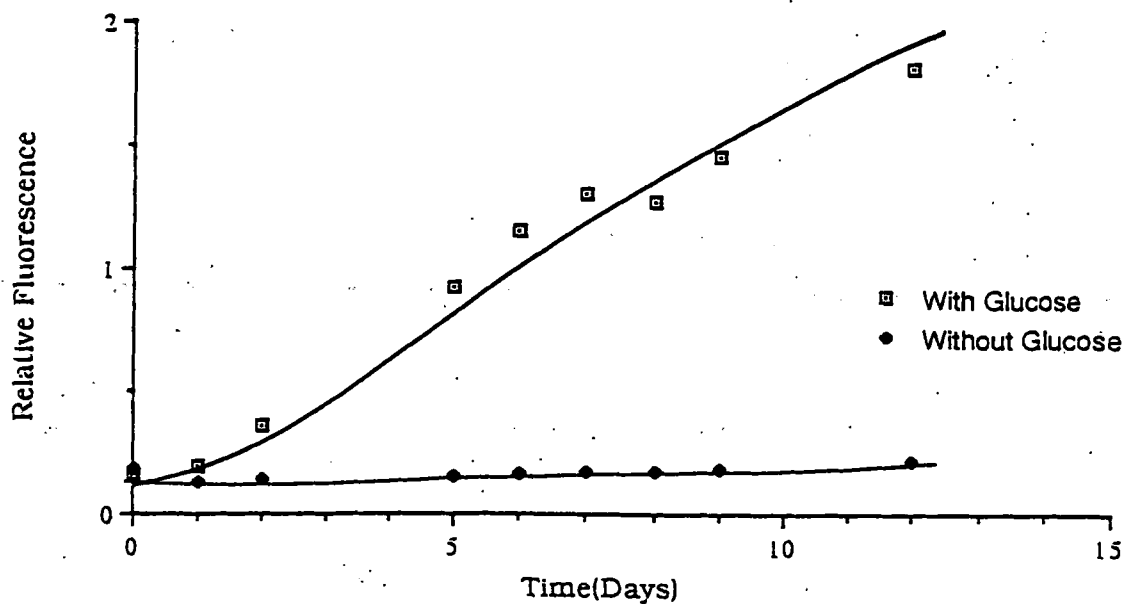


Figure 3.1.1: Fluorescence of native HSA incubated with glucose.

Fluorescence at excitation 325nm and emission 375nm of native HSA incubated at 10mg/ml in 0.2M sodium phosphate buffer (pH 7.4) with glucose (500mM).

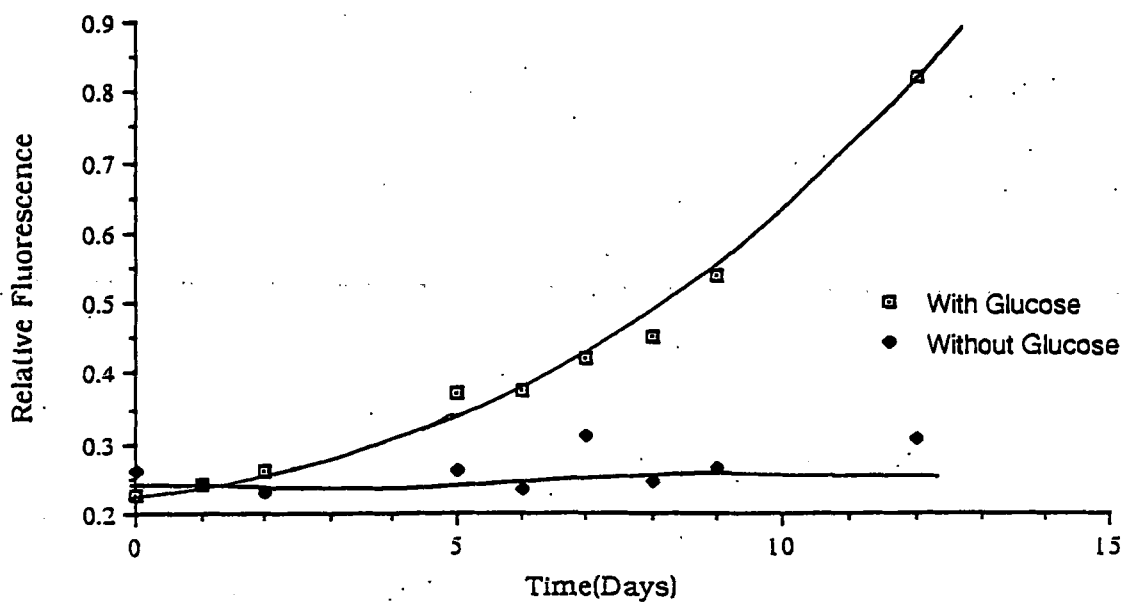


Figure 3.1.2: Fluorescence of delipidated HSA incubated with glucose.

Fluorescence at excitation 325nm and emission 375nm of delipidated HSA incubated at 10mg/ml in 0.2M sodium phosphate buffer (pH7.4) with glucose (500mM).

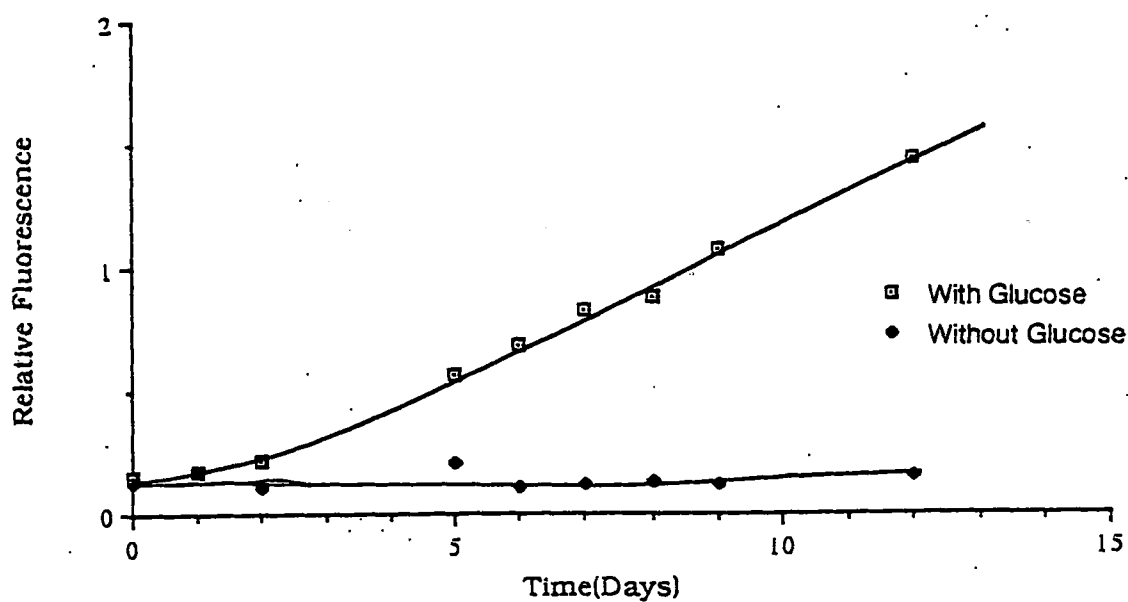


Figure 3.1.3: Fluorescence of recombinant HSA incubated with glucose. Fluorescence at excitation 325nm and emission 375nm of recombinant HSA incubated at 10mg/ml in 0.2M sodium phosphate buffer(pH 7.4) with glucose (500mM).



Figure 3.1.4: Fragmentation of native and delipidated HSA incubated with glucose. Native and delipidated HSA incubated in 0.1M sodium phosphate buffer (pH7.4) for 8 days at 37°C in lanes A and B. Lanes C and D include 250mM glucose in the incubation.

3.2 Effect of protein-bound lipid on the production of Amadori product and AGEs in albumin

The following set of experiments were carried out to determine whether the presence of bound lipid on albumin affected the formation of Amadori product, AGE-type fluorescence and glucose-induced protein fragmentation.

3.2.1 Effect of bound lipid on Amadori product formation

Figure 3.2.1 shows the production of Amadori product on both native and delipidated HSA incubated in 0.05M sodium phosphate buffer, pH7.4 (containing 3mM sodium azide). Glucose was included in the incubation media at concentrations of 0, 50, 100, 200, 300, 400 and 500mM and the incubations were carried out at 37°C over a period of 8 days. The samples were dialysed extensively in the phosphate buffer used in the incubation to remove all unbound glucose, before protein and periodate assays were carried out to determine the number of moles of Amadori product per mole of protein. There appears to be a slight difference between the two forms of HSA in the formation of Amadori product, but the difference is not significant.

3.2.2 Effect of bound fatty acid on fluorescent AGE formation

Figure 3.2.2 shows the relative fluorescence of native, delipidated and recombinant HSA all incubated in 0.2M sodium phosphate buffer (with 3mM NaN₃) at a concentration of 10 mg/ml in the presence and absence of glucose (500mM). It shows that the delipidation of HSA, prior to incubation with glucose, reduces the amount of fluorophore formation during these incubations, even though the starting values for relative fluorescence for native HSA in both its native and delipidated states are very similar. The

delipidated HSA only forms approximately 40% of the amount of fluorescence formed by native HSA when both proteins are incubated under identical conditions. The recombinant HSA has bound lipid of known composition in which a greater proportion of lipids are saturated and shorter chained than those found on native HSA, and it only forms approximately 80% of the fluorescence of native HSA. Even when bound lipid is present on HSA no increase in fluorescence is seen unless glucose is present.

3.2.3 Effect of bound fatty acid on protein fragmentation

In Figure 3.2.3 both native and delipidated HSA are incubated under identical conditions and then examined by SDS-PAGE. It can be seen that for each set of conditions used the fragmentation patterns for both forms of HSA are identical showing that glucose induced fragmentation of HSA is unaffected by the presence of bound lipid on the protein. When both glucose and Cu^{II} are present in the incubation media there is a decrease in the intensity of the 66kD band and there is staining around the 26 - 30 and 15kD areas for both forms of HSA, although the staining of these fragments is very diffuse.

Dog serum albumin (DSA) was included in this study because it has not got the single high affinity binding site for Cu^{II} that is present in HSA. However, the only difference observed in the behaviour of DSA compared to HSA, was with respect to its bound lipid. Figure 3.2.4 shows the extent of fragmentation of native and delipidated DSA that has been incubated under identical conditions. The fragmentation of the two forms of DSA is very similar to each other except when glucose and Cu^{II} are added to the incubation (lanes D and H respectively). Delipidated DSA fragments to a greater extent than native DSA as judged by the decrease in intensity of the 66kD band and an increase in smearing. Both the native and delipidated DSA produce a fragment of approximately 26 - 30kD and like the fragments found in Figure 3.2.3 it appears as a diffuse band.

3.2.4 Aggregation

Incubating native HSA in the presence of FeCl_3 (Figure 3.4.12) produces no fragmentation but it does appear to promote the production of high molecular mass oligomers. This effect is only seen for HSA with bound lipids, i.e. native HSA. No increase in high molecular mass substances is seen in the incubation of delipidated HSA (Figure 3.4.13).

3.2.5 Direct incubation of fatty acid with protein and glucose

Direct incubation of native BSA with lipid and glucose was attempted following work carried out by Birruez-Aragon et al (personal communication). Native BSA was incubated at 40°C with linoleic acid at a ratio of 10 moles of lipid per mole of BSA, and the fluorescence at 325/375 and 370/440 was followed. After incubation for 78 minutes the glucose concentration was increased to 250mM to see whether the higher glucose concentration would cause an increase in fluorescence. After 160 minutes the incubation was halted as no appreciable increase in fluorescence was observed and it was seen that the lipid was no longer mixing with the protein, but was floating on top of the incubation media.

3.2.6 Summary

The results from Sections 3.2.2 and 3.2.3 indicate that the mechanisms for the formation of AGE-type fluorescence and for the glucose-induced fragmentation of HSA are different. The fatty acids bound to HSA appear not to affect the fragmentation of the protein at all, but the delipidated HSA only produces approximately 40% of the amount of fluorescence produced by HSA with bound fatty acids under the conditions used in these experiments. However, if a HEPES buffer system is used instead of phosphate

there is very little difference between the amount of fluorophore production by the different forms of HSA (see Section 3.5, Figure 3.5.4). In DSA, unlike HSA, the presence of bound lipid does affect the fragmentation of the protein with the delipidated DSA being fragmented to a greater extent than native DSA. The production of Amadori product on HSA does not appear to be affected by the presence of bound lipids.

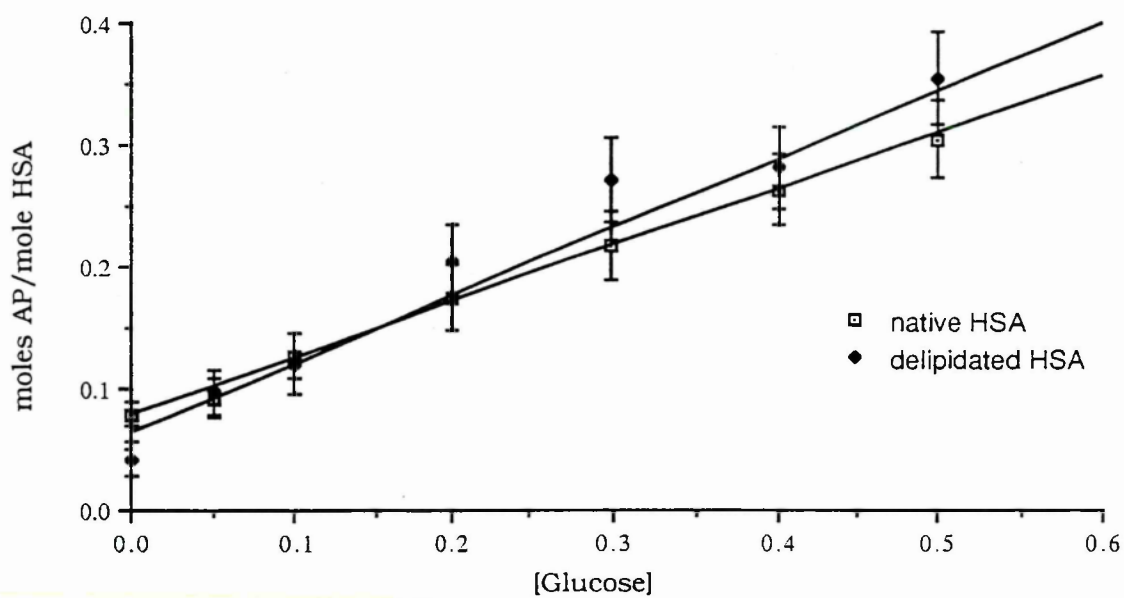


Figure 3.2.1: Production of Amadori product in native and delipidated HSA.

Native and delipidated HSA were incubated with glucose (0, 50, 100, 200, 300, 400 and 500mM) for 8 days in 0.1M sodium phosphate buffer.

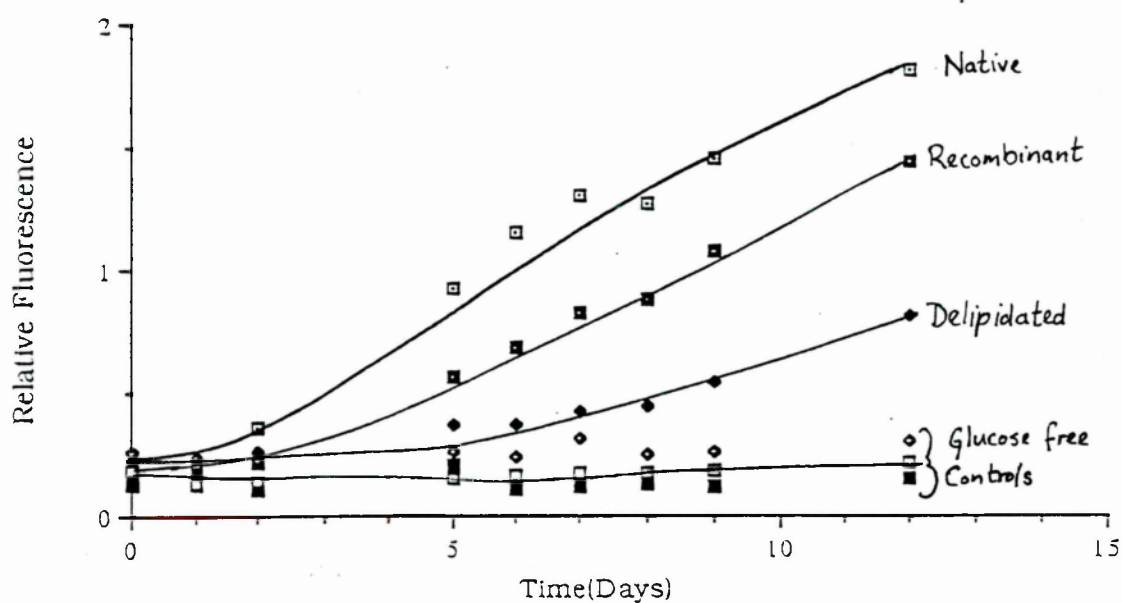


Figure 3.2.2: Fluorescence of native, delipidated and recombinant HSA.

Fluorescence at excitation 325nm and emission 375nm of native, delipidated and recombinant HSA all incubated at 10mg/ml in 0.2M sodium phosphate buffer, pH 7.4, with glucose (500mM).

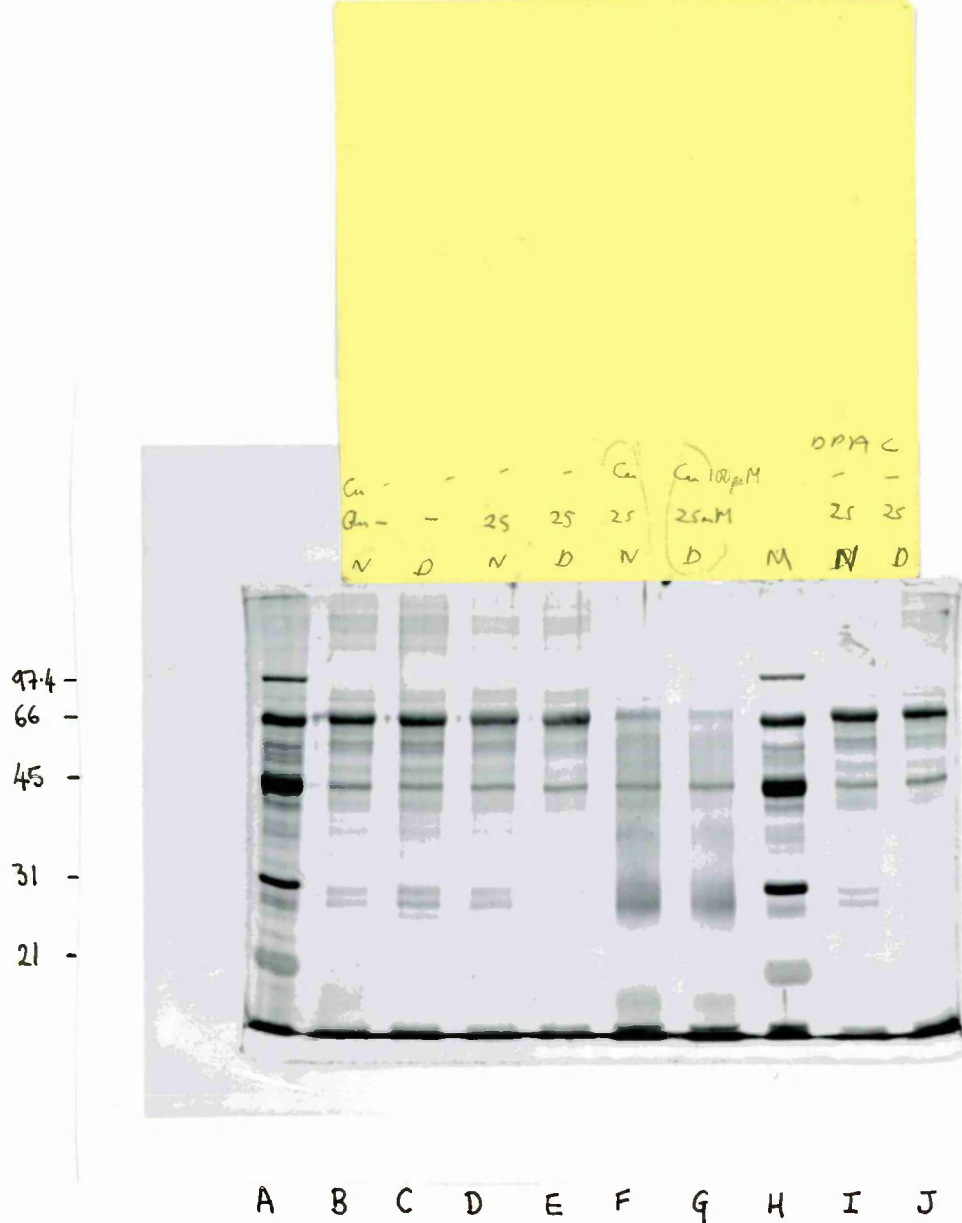


Figure 3.2.3: Fragmentation of native and delipidated HSA incubated with glucose, copper and DTPA. Lanes B and C show native and delipidated HSA incubated for 8 days at 37°C in 0.1M sodium phosphate buffer (pH7.4) respectively. Lanes D and E show the effect of glucose (25mM), lanes F and G show the effect of glucose and CuSO₄ (100 μ M), and lanes I and J show the effect of glucose and DTPA (1mM), on the two forms of HSA.

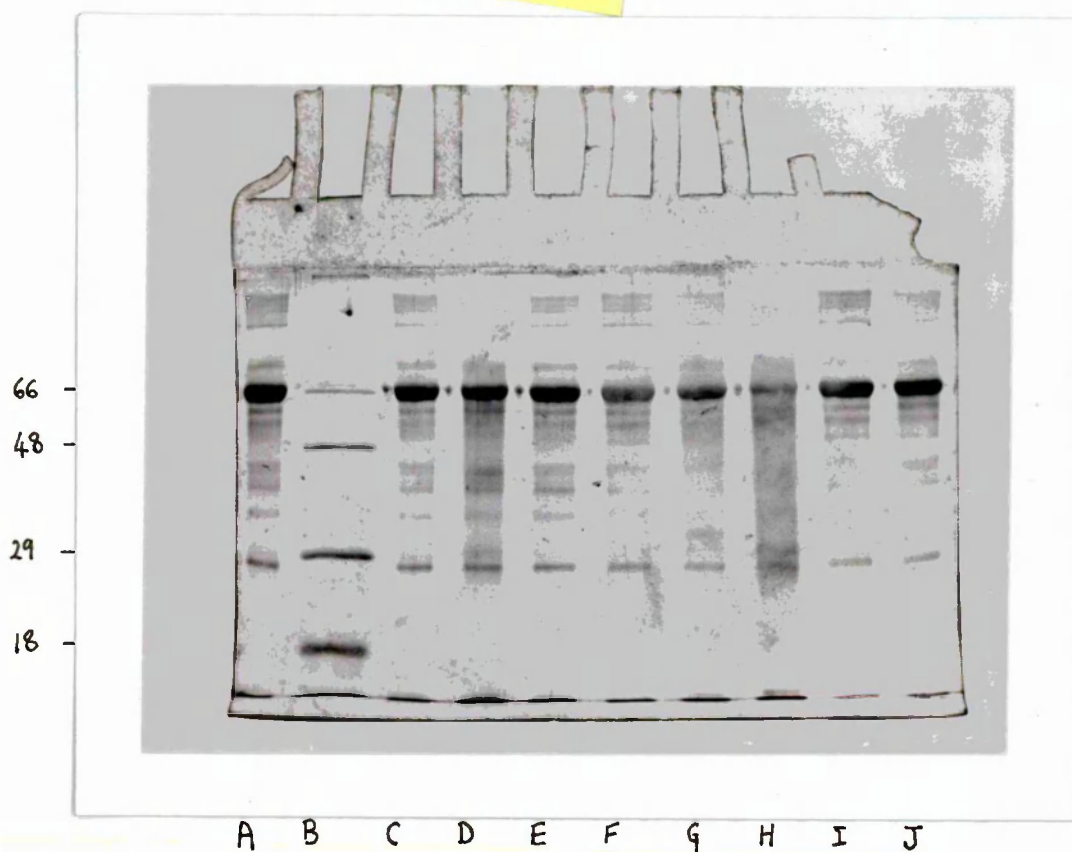


Figure 3.2.4: Fragmentation of native and delipidated DSA incubated with glucose, copper and DTPA. Native DSA (lanes A, C, D and E) and delipidated DSA (lanes F, G, H, I and J) were incubated for 8 days at 37°C in 0.1M sodium phosphate buffer, pH 7.4, with glucose (25mM) in lanes C and G, with glucose and CuSO₄ (100µM) in lanes D and H, with glucose and DTPA (1mM) in lane I and with CuSO₄ (100µM) in lanes E and J.

3.3 Effect of trace transition metals on AGE formation

Traces of the transition metals copper and iron are present even in high grade preparations of sodium phosphate buffers. It was therefore decided to determine whether these contaminants play a role in the production of AGEs by using the metal chelator diethylenetriaminepentacetic acid (DTPA).

3.3.1 Fluorescence

The transition metal chelator DTPA was added to the incubation media to test whether traces of transition metals in the incubation media were catalysing the development of fluorescent AGEs. Figures 3.3.1, 3.3.2 and 3.3.3 show the effect of adding DTPA (1mM) to the incubation media used in Figures 3.1.1, 3.1.2 and 3.1.3. The glucose-free controls for each of the three forms of HSA appear as virtually identical traces whether DTPA was present in the media or not. The presence of DTPA reduces the amount of glycofluorophore formation compared to when the proteins are incubated with glucose alone. The inhibition of fluorescence formation was 88% for native HSA, 100% for delipidated HSA and 63% for recombinant HSA when DTPA was present at a concentration of 1mM. The approximate concentration of transition metal contaminants in the phosphate buffer as calculated from the suppliers analysis are 4 μ M Cu^{II} and 5 μ M Fe^{III}. All solutions were made with high quality distilled water, so that any additional transition metal contaminants from this source were at such low concentrations that the metal chelator DTPA was in very large excess.

3.3.2 Fragmentation

Fragmentation gels of transferrin, native HSA, delipidated HSA, native DSA and delipidated DSA all incubated in the presence of glucose with and without DTPA show that DTPA in the incubation media can inhibit the fragmentation of all of these proteins. Figure 3.3.4 shows the effect of DTPA on the fragmentation of both native HSA and delipidated HSA. Lanes D and E show both native and delipidated HSA incubated with glucose, and lanes I and J show the same samples with DTPA also included in the incubation. The samples incubated with DTPA appear to be very similar to the glucose free controls run in lanes B and C. In lanes D and E there is a marked decrease in the intensity of the 66kD band relative to the 45kD marker band intensity, showing that protein fragmentation is occurring in the presence of glucose unless DTPA is present. The inhibition of protein fragmentation by DTPA is not confined to HSA. Both transferrin (Figure 3.4.6 - lane E) and DSA (Figure 3.2.4 - lane I) show complete inhibition of protein fragmentation when DTPA is included.

3.3.3 Summary

Both the fluorescence and fragmentation results show that AGE production is inhibited by the presence of DTPA, showing that these reactions are transition metal catalysed. However, the inhibition of fluorescence formation in recombinant and native HSA is not 100% under the conditions used, although there is 100% inhibition of fluorophore production in the delipidated HSA.

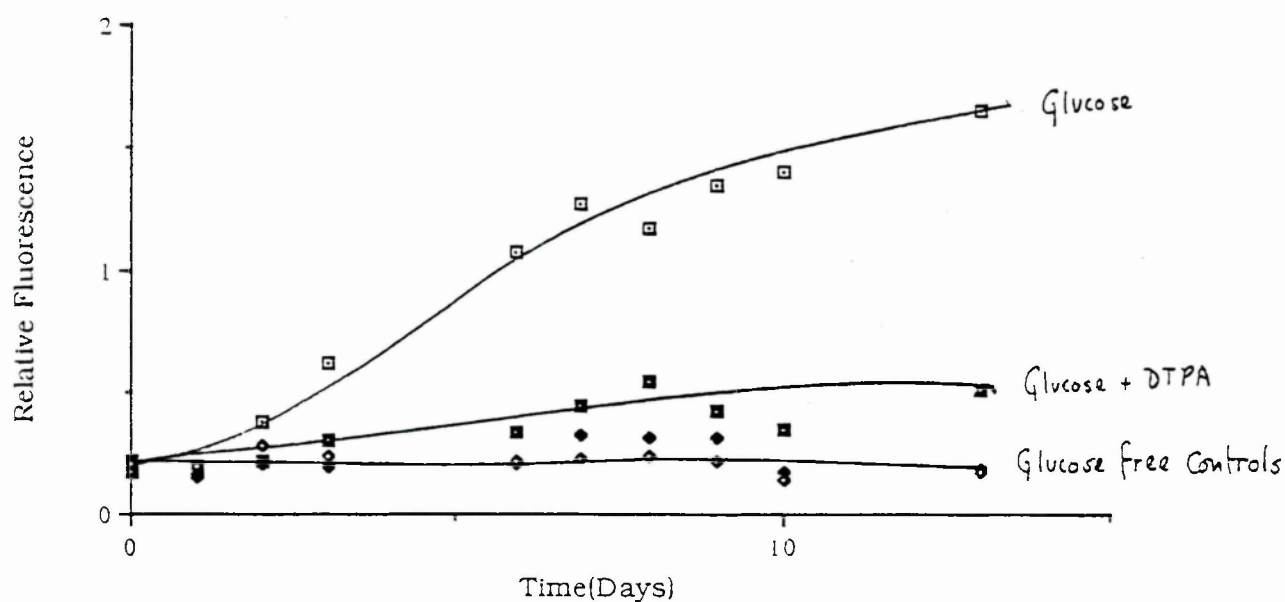


Figure 3.3.1: Fluorescence of native HSA incubated with glucose and DTPA.

Fluorescence at excitation 325nm and emission 375nm of native HSA incubated at 10mg/ml in 0.2M sodium phosphate buffer (pH 7.4) with glucose (500mM) and DTPA (1mM).

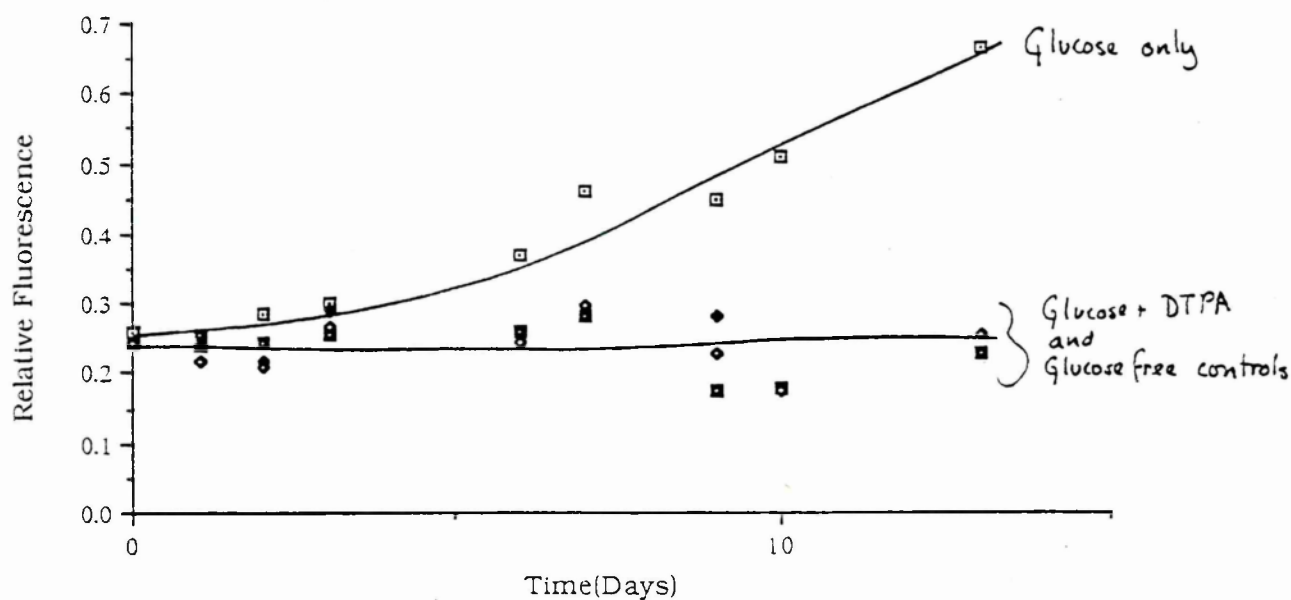


Figure 3.3.2: Fluorescence of delipidated HSA incubated with glucose and DTPA.

Fluorescence at excitation 325nm and emission 375nm of delipidated HSA incubated at 10mg/ml in 0.2M sodium phosphate buffer (pH 7.4) with glucose (500mM) and DTPA (1mM).

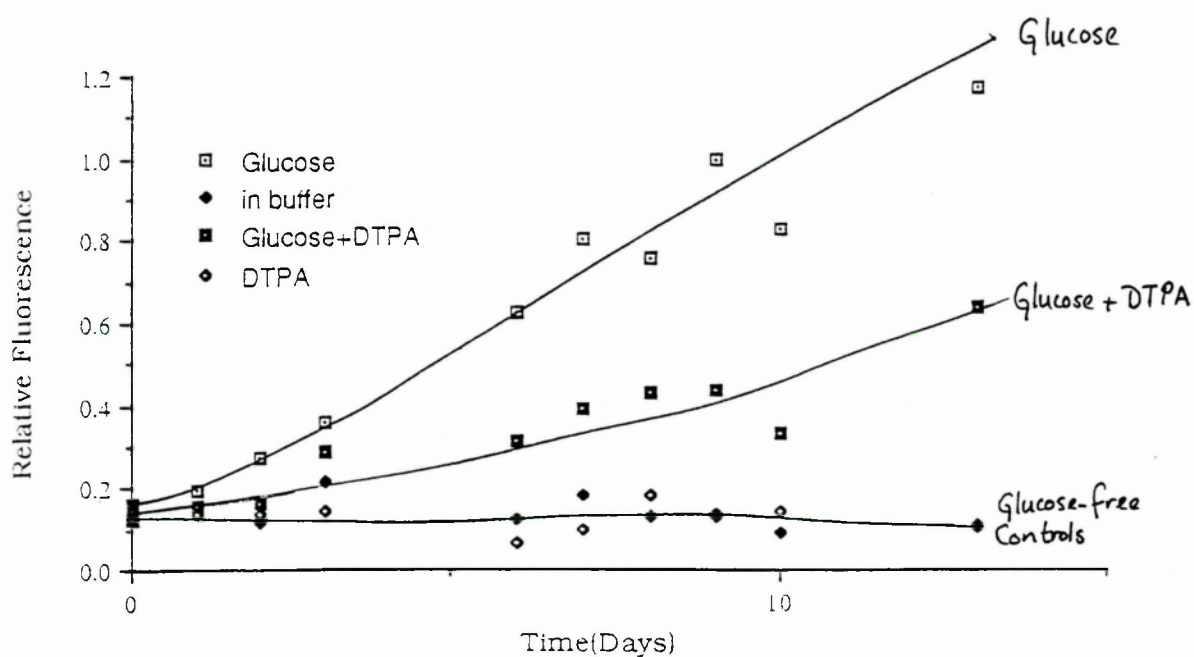


Figure 3.3.3: Fluorescence of recombinant HSA incubated with glucose and DTPA. Fluorescence at excitation 325nm and emission 375nm of recombinant HSA incubated at 10mg/ml in 0.2M sodium phosphate buffer (pH 7.4) with glucose (500mM) and DTPA (1mM).

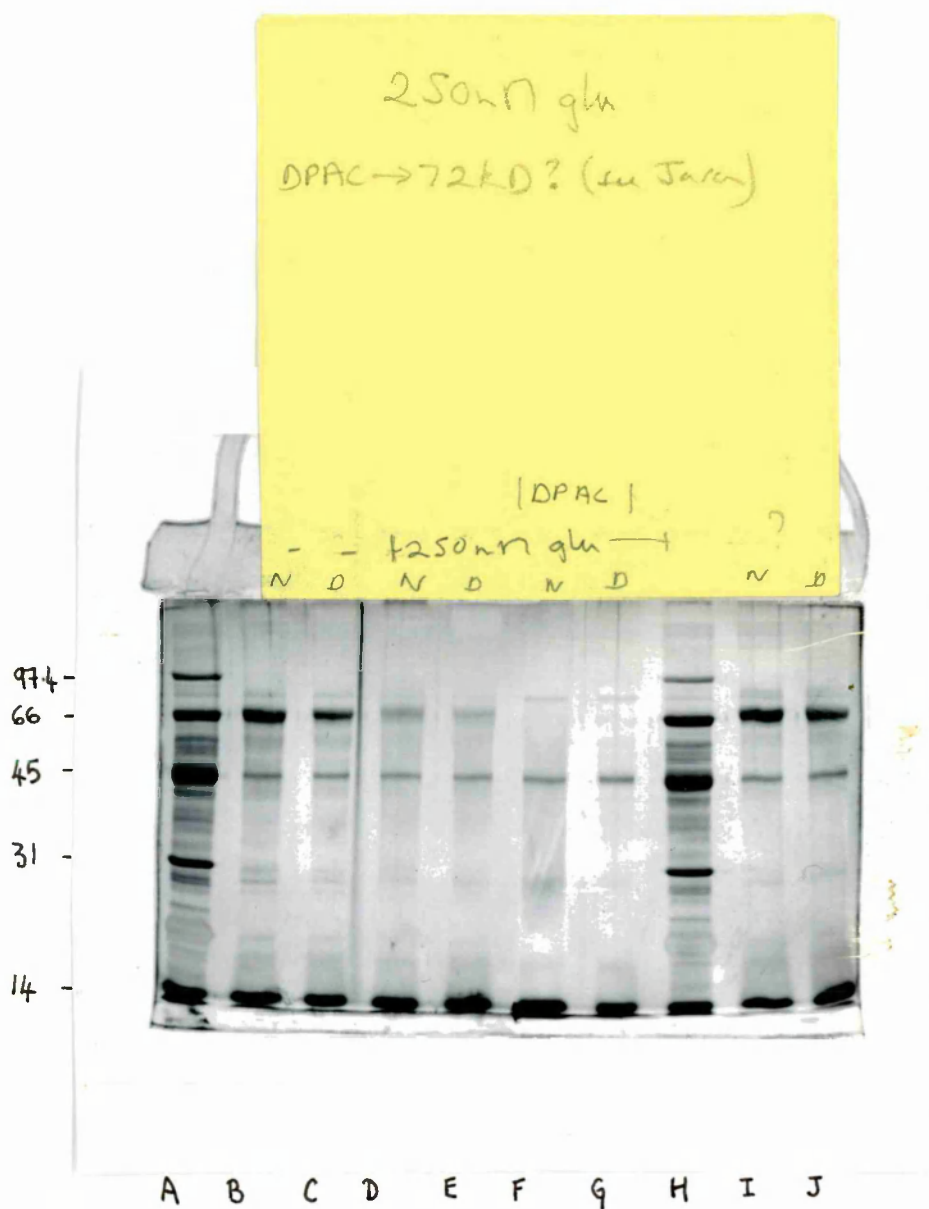


Figure 3.3.4: Fragmentation of native and delipidated HSA incubated with glucose and DTPA. Native and delipidated HSA were incubated at 1mg/ml in 0.1M sodium phosphate buffer, pH 7.4, at 37°C for 8 days. Lanes B and C show native and delipidated HSA incubated in buffer, Lanes D and E the effect of glucose (250mM), and lanes F and G show the effect DTPA (1mM) and glucose (250mM) have on the fragmentation of the two proteins respectively.

3.4 Effects of the transition metals Cu^{II} and Fe^{III} on AGE formation

Traces of the both Cu^{II} and Fe^{III} (approximately 4μM and 5μM respectively) are found in the 0.1M sodium phosphate buffer used in earlier experiments. The inclusion of DTPA in this buffer system inhibits the formation of AGEs so the effect of adding further quantities of each of these metals was investigated.

3.4.1 Effect of Cu^{II} on AGE fluorescence formation

Figure 3.4.1 shows native, delipidated and recombinant HSA incubated under identical conditions to Figure 3.2.2 (i.e. with 500mM glucose), apart from the inclusion of 50μM CuSO₄ in the incubation media. By comparing these two figures, it can be seen that adding copper has no effect on the fluorescence of native or delipidated HSA, but enhances the fluorescence of recombinant HSA.

Figures 3.4.2, 3.4.3 and 3.4.4 show the fluorescence of native and delipidated HSA and transferrin all incubated at 1mg/ml in 0.1M sodium phosphate buffer (pH7.4) at 37°C for 8 days with glucose (25mM) and increasing concentrations of CuSO₄ (0, 10, 25, 50, 100 and 200μM). The concentration of glucose used in this experiment was very much lower than that shown in Figure 3.4.1; 25mM as opposed to 500mM. Fluorescence was measured at both excitation 325nm/emission 375nm and excitation 370nm/emission 440nm. The two wavelengths follow the same trend for all three proteins, hence only the excitation 325nm/emission 375nm results are shown.

Both native HSA (Figure 3.4.2) and delipidated HSA (Figure 3.4.3) show an increase in fluorescence when the added copper concentration is above 10μM (in addition to the trace contaminants in the phosphate buffer). At concentrations of added copper at and

below 10 μ M little increase in fluorophore production is observed. The increase in fluorophore production is dose dependent for 50, 100 and 200 μ M of added Cu^{II}.

Figure 3.4.4 shows that very little fluorophore is produced in transferrin even when the concentration of copper ions is as high as 100 μ M. However, when 200 μ M copper is used, there is a slight increase in fluorescence.

3.4.2 Effect of Cu^{II} on sugar induced protein fragmentation

The effect of added Cu^{II} ions on the fragmentation of HSA can be seen in Figure 3.4.5. There is very little fragmentation of HSA under this set of experimental conditions unless additional Cu^{II} is present. Lanes C and D show native HSA incubated with glucose, with and without Cu^{II} respectively. Lanes G and H show delipidated HSA incubated under the same conditions. There is a very large increase in the amount of protein fragmentation in the two samples which have Cu^{II} included (lanes D and H). As in Figure 3.2.3, the fragmentation is seen mostly as a smear with diffuse bands appearing at approximately 15 and 26 - 33kD. The samples incubated with Cu^{II} in the absence of glucose show no fragmentation (lanes E and J). In Figure 3.3.4 the same proteins showed significant fragmentation in the absence of added Cu^{II}, but the concentration of glucose in that incubation is 10-fold higher than that used in this experiment.

Figure 3.4.6 shows human transferrin which has been incubated with glucose (lane C) and both glucose and Cu^{II} (lane D). Several discrete fragments form in the incubation which includes added Cu^{II} and glucose, but no fragmentation is seen in the sample incubated with glucose alone. The masses of these discrete fragments have been calculated from a log plot to be around 62, 41, 32 and 16kD.

A dose-dependent increase in fragmentation of native HSA (Figure 3.4.7) and transferrin (Figure 3.4.8) is seen when increasing concentrations of Cu^{II} are added to the

incubations. For native HSA the intensity of the smearing increases with Cu^{II} concentration, although no fragmentation can be observed where the Cu^{II} concentrations are below $50\mu\text{M}$. The transferrin fragments of 32 and 41kD increase in intensity as Cu^{II} concentration increases. This effect is seen throughout the range of Cu^{II} concentrations used. However, even at the maximum concentration of Cu^{II} used ($200\mu\text{M}$) the fragmentation of both proteins is not as advanced as in Figures 3.4.5 and 3.4.6.

3.4.3 Effect of Fe^{III} on the production of fluorescent AGEs

The effect that free Fe^{III} ions have on the development of fluorescent AGEs was investigated by incubating both native and delipidated HSA and transferrin in 0.1M sodium phosphate buffer (pH7.4). Proteins were incubated at 1mg/ml with 25mM glucose and increasing concentrations of FeCl_3 . These incubations were at 37°C over a period of 8 days.

Native HSA shows a slight increase in fluorophore production when iron chloride is added to the incubation medium up to a concentration of $10\mu\text{M}$. Above $10\mu\text{M}$ FeCl_3 the increase in the amount of fluorophore production is very small. The delipidated HSA (Figure 3.4.10) shows an increase in fluorescence when $10\mu\text{M}$ FeCl_3 is added to the incubation, but the fluorescence does not appear to increase with further increases in the concentration of FeCl_3 .

3.4.4 Effect of Fe^{III} on sugar induced protein fragmentation

In Figures 3.4.12 and 3.4.13, both native and delipidated HSA were incubated with increasing concentrations of iron chloride in the presence of 25mM glucose. Fe^{III} does not appear to induce protein fragmentation in native HSA (Figure 3.4.12), delipidated HSA (Figure 3.4.13) or transferrin (Figure 3.4.6, lane F). Unlike Figure 3.4.7, where dose-dependent fragmentation of native HSA is seen with increasing concentrations of Cu^{II} ions, no fragmentation is observed even when the concentration of Fe^{III} ions is 200μM. Native HSA (Figure 3.4.12) showed slight production of high molecular mass substances when high concentrations of FeCl₃ were included in the incubation. There was no sign of these high molecular mass substances in incubations of delipidated HSA (Figure 3.4.13). Transferrin (Figure 3.4.6) incubated with glucose and Fe^{III} (lane F) did not fragment, unlike transferrin incubated with glucose and Cu^{II} (lane D).

3.4.5 Summary

When a high concentration of glucose was used, the fluorescence of native and delipidated HSA was unaffected by the addition of 100μM CuSO₄ to the incubation media, but recombinant HSA showed an increase in the fluorescence. However, at low concentrations of glucose there was an increase in fluorescence in both native and delipidated HSA when only 50μM CuSO₄ was added.

The fragmentation of native and delipidated HSA can occur in the absence of added copper if glucose concentrations are high (Figure 3.2.3), but when a lower concentration of glucose was used, added copper was needed for fragmentation to occur.

Transferrin produced glycofluorophores in the presence of FeCl₃, but when CuSO₄ was used, there was only a relatively small increase in fluorescence. However, fragmentation of transferrin (which produces four discrete fragments) occurs in the presence of CuSO₄, but not FeCl₃.

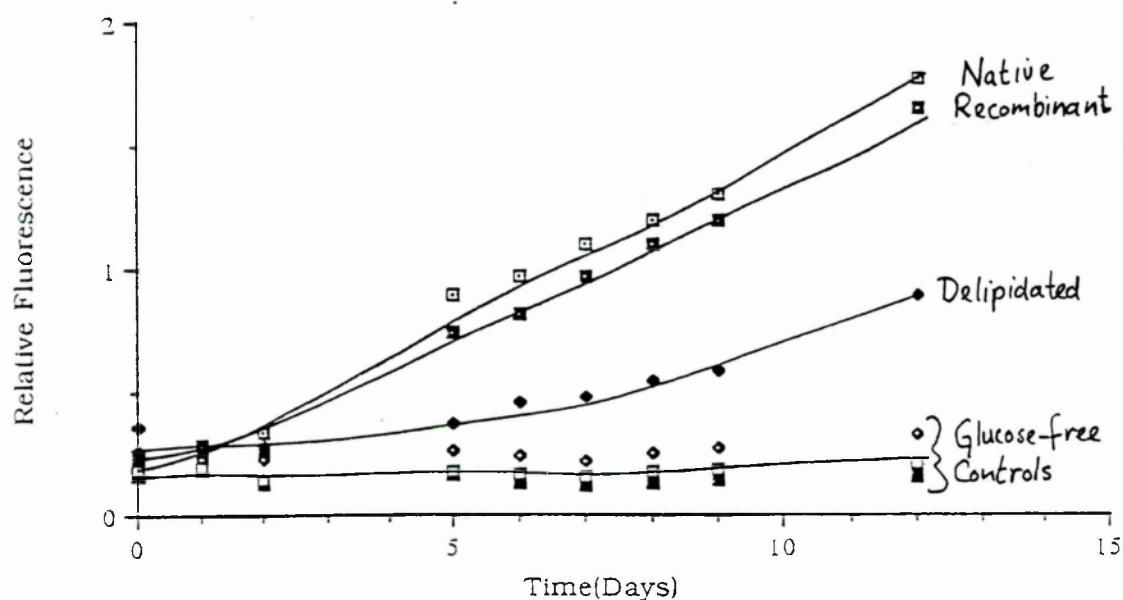


Figure 3.4.1: Fluorescence of native, delipidated and recombinant HSA incubated with glucose and copper. Fluorescence at excitation 325nm and emission 375nm of native, delipidated and recombinant HSA all incubated at 10mg/ml in 0.2M sodium phosphate buffer, pH 7.4, with glucose (500mM) and CuSO_4 (50 μM).

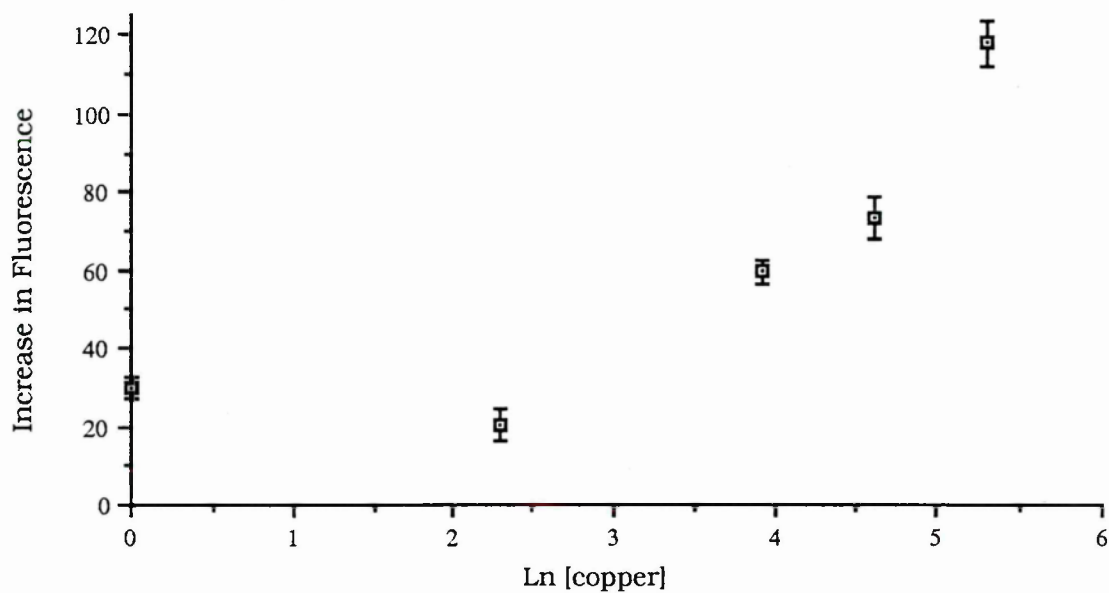


Figure 3.4.2: Fluorescence of native HSA incubated with glucose and increasing concentrations of copper. Fluorescence at excitation 325nm and emission 375nm of native HSA incubated in 0.1M phosphate buffer for 8 days with glucose (25mM) and increasing concentrations of CuSO_4 (0, 10, 50, 100 and 200 μM) added to the incubation media. Error bars show 1 standard deviation, where $n=3$.

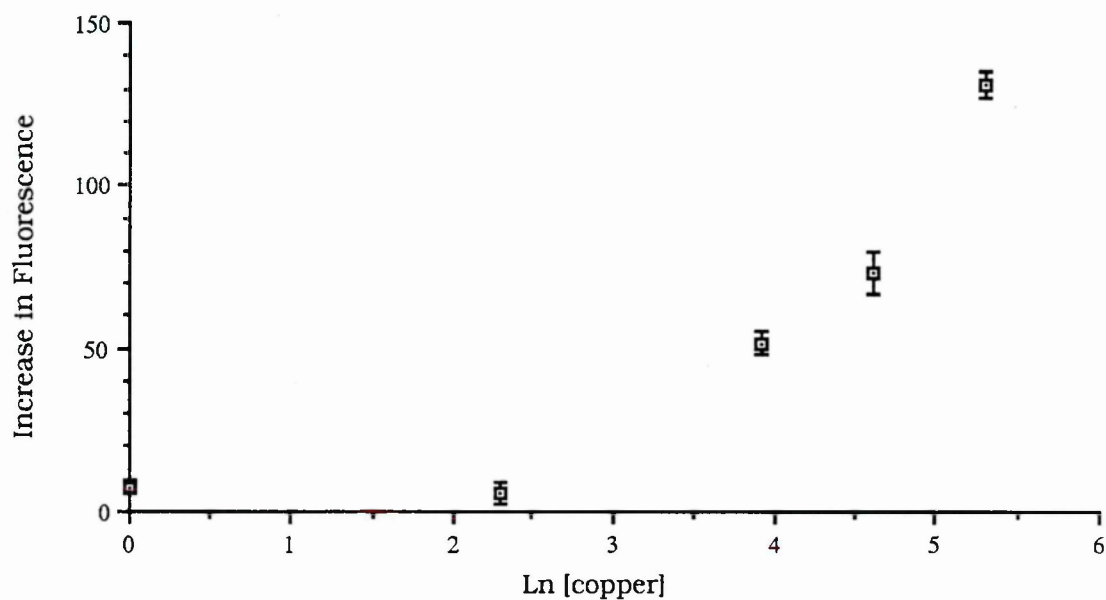


Figure 3.4.3: Fluorescence of delipidated HSA incubated with glucose and increasing concentrations of copper. Fluorescence at excitation 325nm and emission 375nm of delipidated HSA incubated in 0.1M phosphate buffer for 8 days with glucose (25mM) and increasing concentrations of CuSO_4 (0, 10, 50, 100 and 200 μM) added to the incubation media. Error bars show 1 standard deviation, where $n=3$.

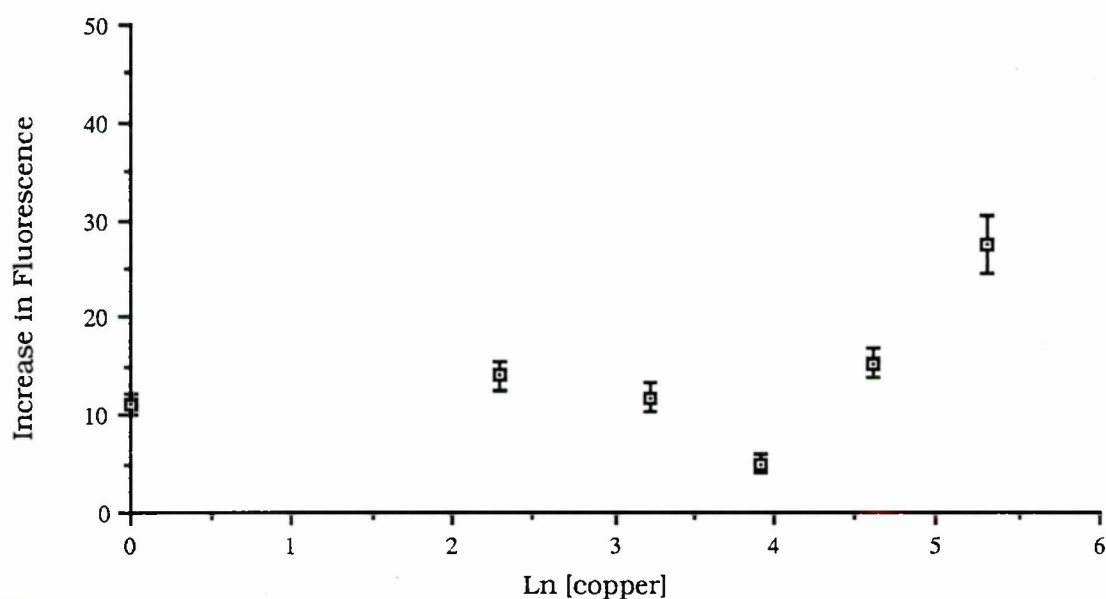


Figure 3.4.4: Fluorescence of transferrin incubated with glucose and increasing concentrations of copper. Fluorescence at excitation 325nm and emission 375nm of transferrin incubated in 0.1M phosphate buffer for 8 days with glucose (25mM) and increasing concentrations of CuSO_4 (0, 10, 25, 50, 100 and 200 μM) added to the incubation media. Error bars show 1 standard deviation, where $n=3$.

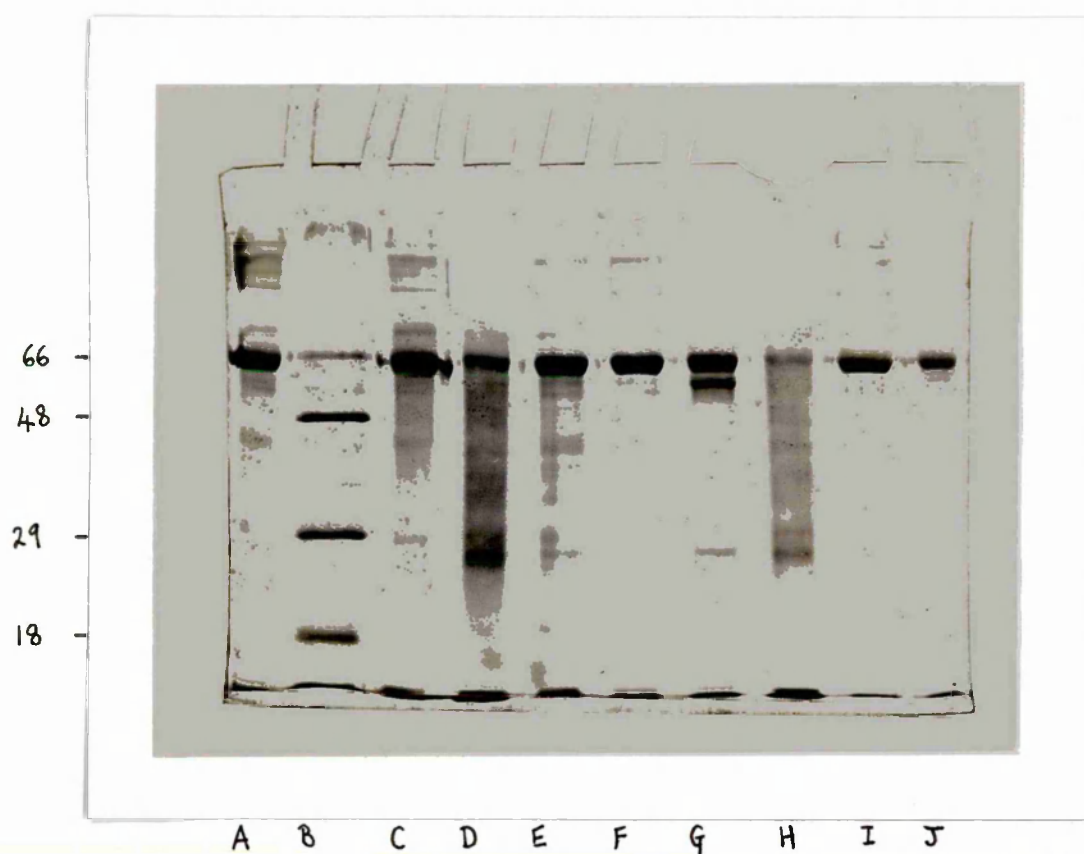


Figure 3.4.5: Fragmentation of native and delipidated HSA incubated with glucose and copper. Native HSA (lanes A,C,D and E) and delipidated HSA (lanes F,G,H,I and J) were incubated for 8 days in 0.1M sodium phosphate buffer with glucose (25mM) in lanes C and G, with glucose and CuSO_4 (100 μM) in lanes D and H and with CuSO_4 in lanes E and J.

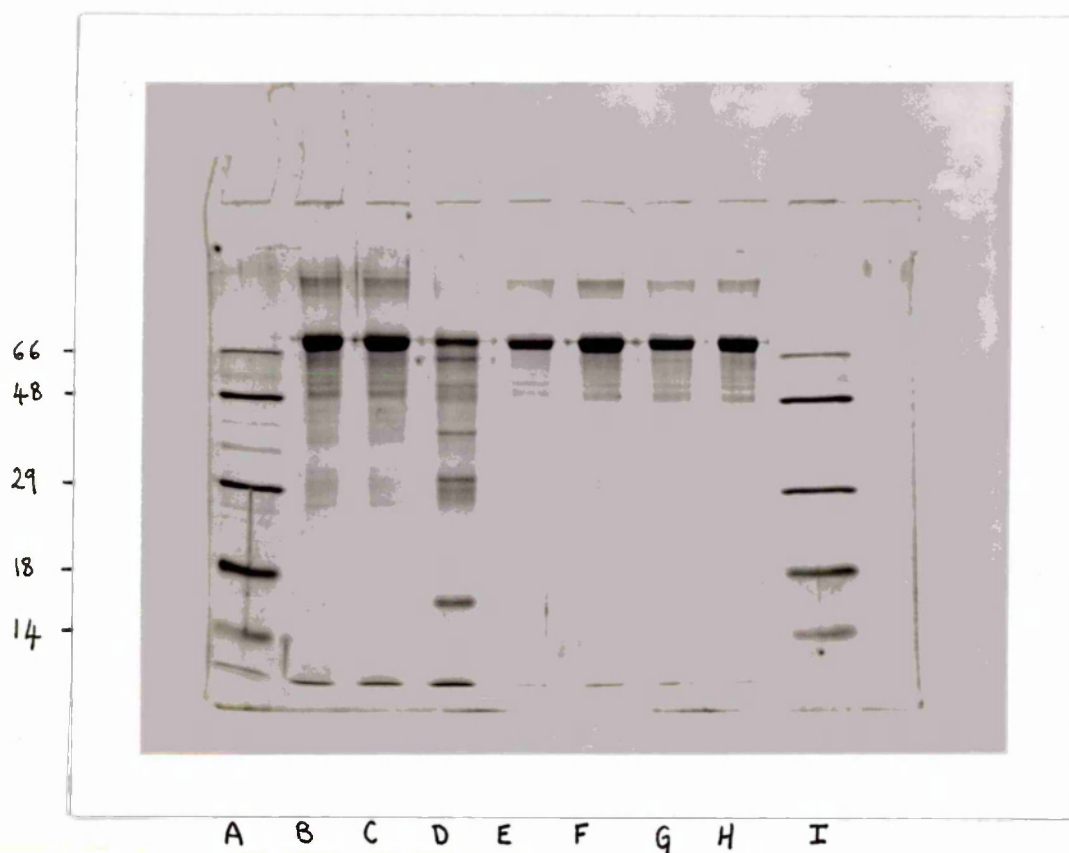


Figure 3.4.6: Fragmentation of transferrin incubated with glucose, copper, iron and DTPA. Transferrin was incubated in 0.1M sodium phosphate buffer for 8 days (lane B). The incubation media included glucose (25mM) in lane C, glucose and CuSO_4 (100 μM) in lane D, glucose and DTPA (1mM) in lane E, glucose and FeCl_3 (100 μM) in lane F and CuSO_4 and FeCl_3 alone in lanes G and H respectively.

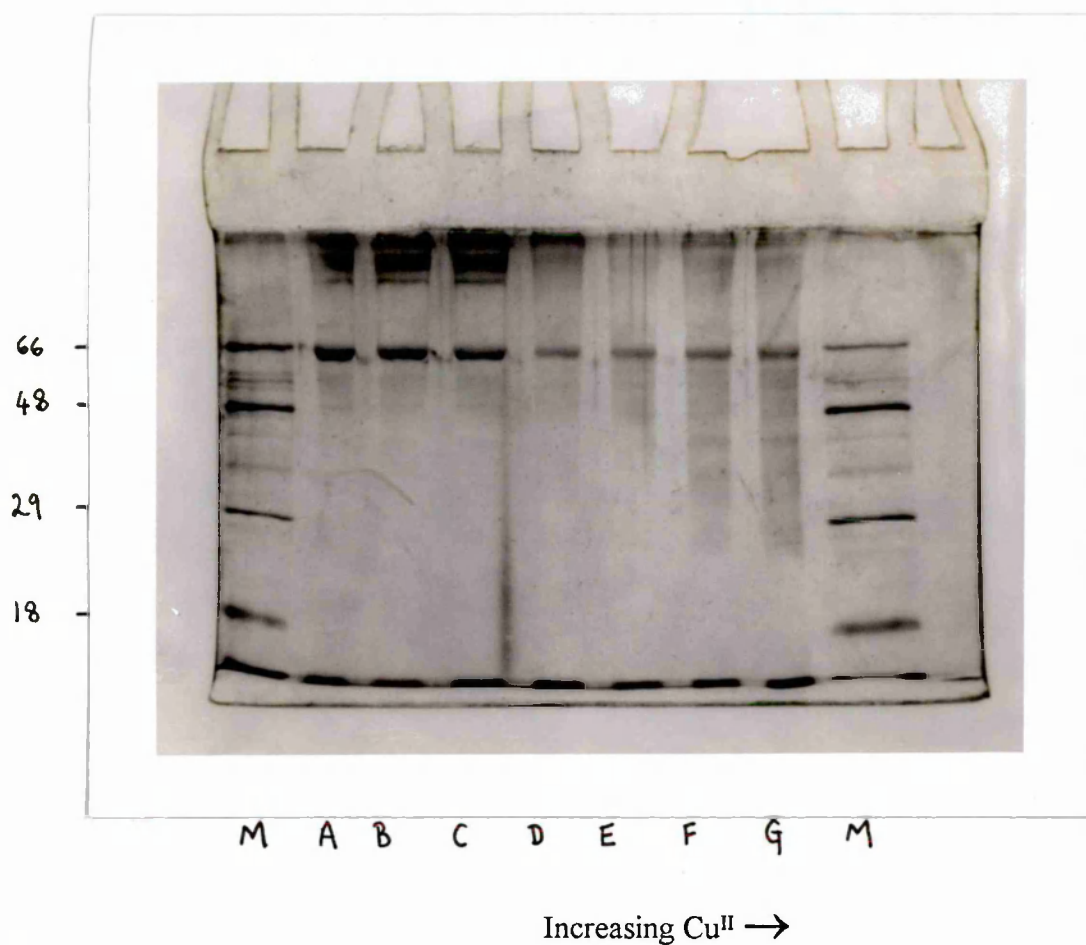
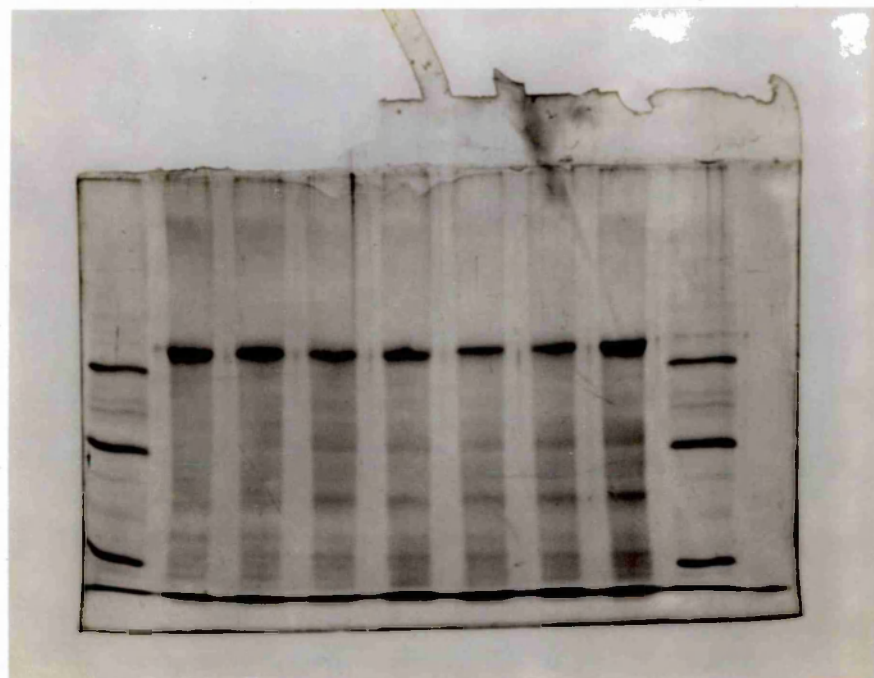


Figure 3.4.7: Fragmentation of native HSA incubated with glucose and increasing concentrations of copper. Lanes A and B are native HSA incubated alone and with 25mM glucose respectively. Lanes C to G have native HSA incubated with glucose and copper sulphate at concentrations of 10, 25, 50, 100 and 200 μ M respectively.

Increase in Fluorescence

1
2
3
4
5
6
7
8
9
10

66
48
18



M A B C D E F G M

Increasing Cu^{II} \rightarrow

Fi

co

HS

and

shc

Figure 3.4.8: Fragmentation of transferrin incubated with glucose and increasing concentrations of copper. Lanes A and B are transferrin incubated alone and with glucose respectively. Lanes C to G have transferrin incubated with glucose and copper sulphate at concentrations of 10, 25, 50, 100 and 200 μM respectively.

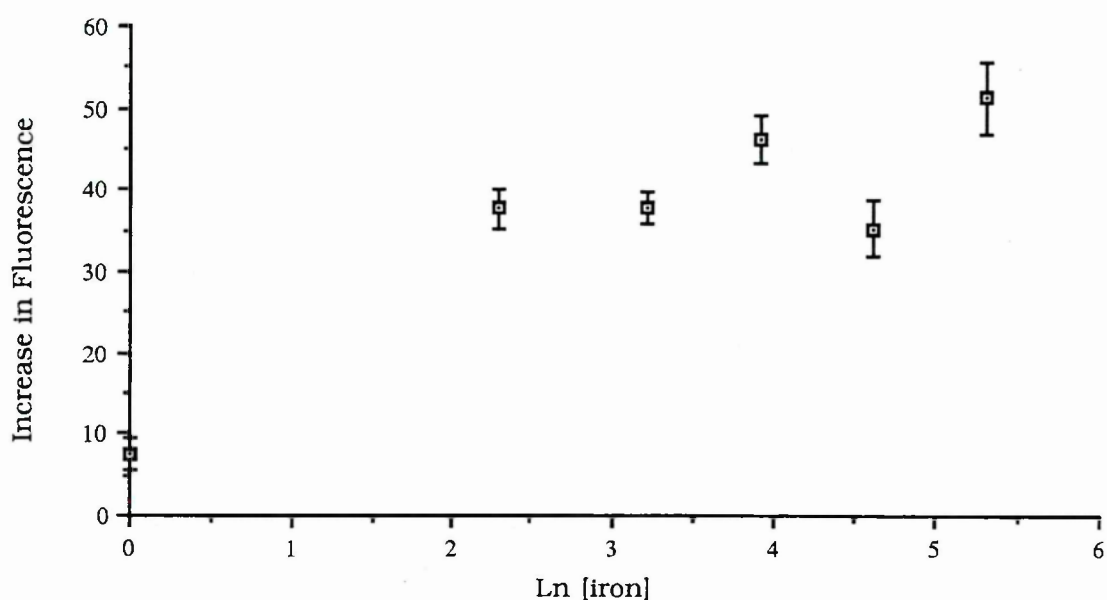


Figure 3.4.10: Fluorescence of delipidated HSA incubated with glucose and increasing concentrations of iron. Fluorescence at excitation 325nm and emission 375nm of delipidated HSA incubated in 0.1M sodium phosphate buffer (pH7.4) for 8 days with 25mM glucose and increasing concentrations of added FeCl_3 (0, 10, 25, 50, 100 and 200 μM). Error bars show 1 standard deviation where $n=3$.

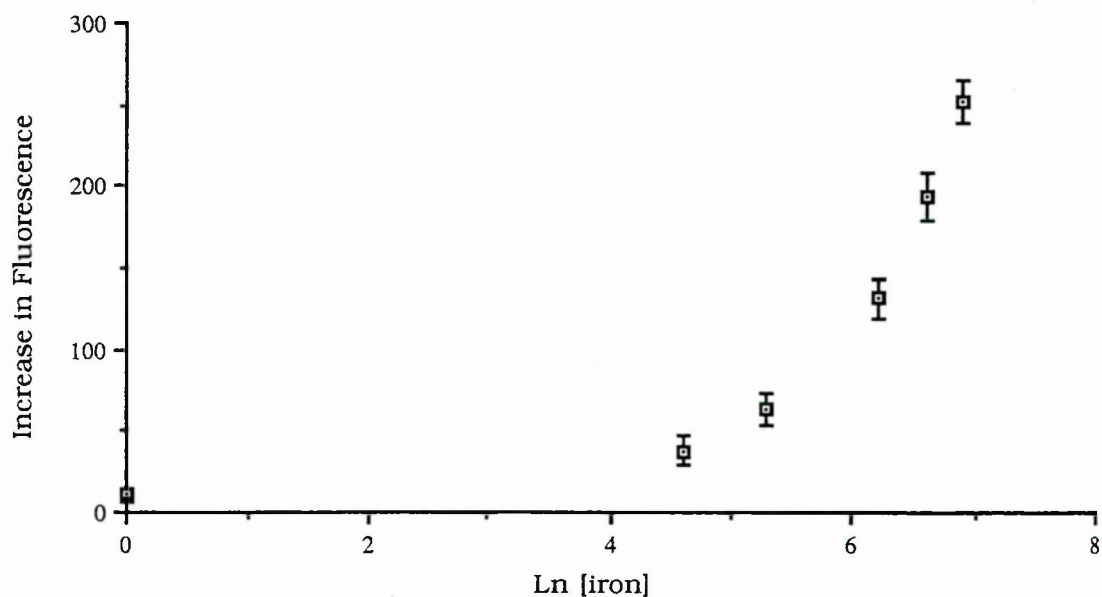


Figure 3.4.11: Fluorescence of transferrin incubated with glucose and increasing concentrations of iron. Fluorescence at excitation 325nm and emission 375nm of transferrin incubated in 0.1M sodium phosphate buffer (pH7.4) for 8 days with 25mM glucose and increasing concentrations of added FeCl_3 (0, 100, 200, 500, 750 and 1000 μM). Error bars show 1 standard deviation where $n=3$.

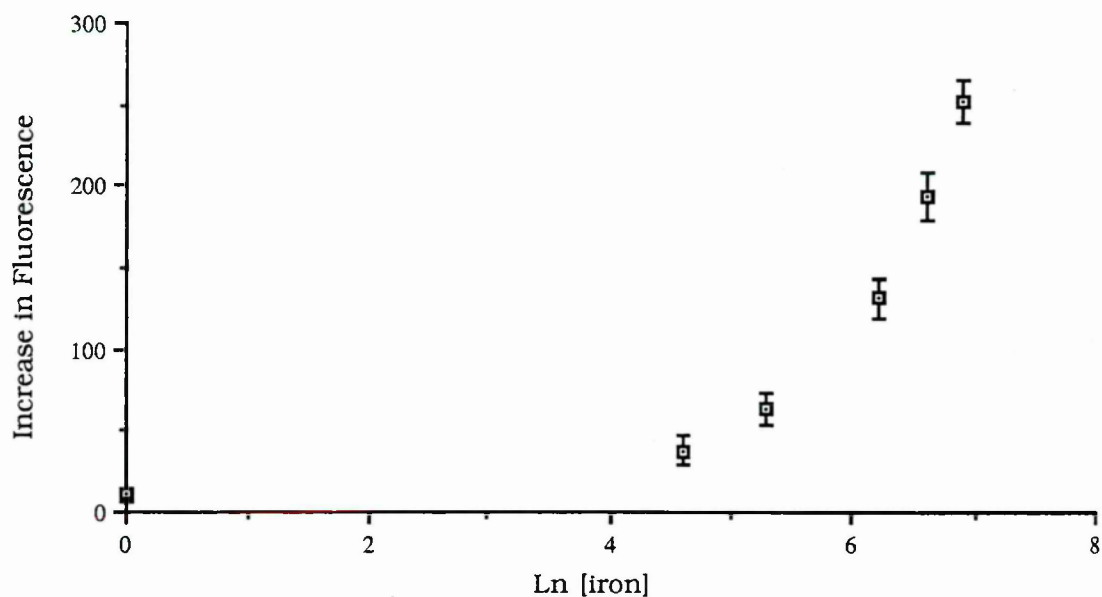


Figure 3.4.11: Fluorescence of transferrin incubated with glucose and increasing concentrations of iron. Fluorescence at excitation 325nm and emission 375nm of transferrin incubated in 0.1M sodium phosphate buffer (pH7.4) for 8 days with 25mM glucose and increasing concentrations of added FeCl_3 (0, 100, 200, 500, 750 and 1000 μM). Error bars show 1 standard deviation where $n=3$.

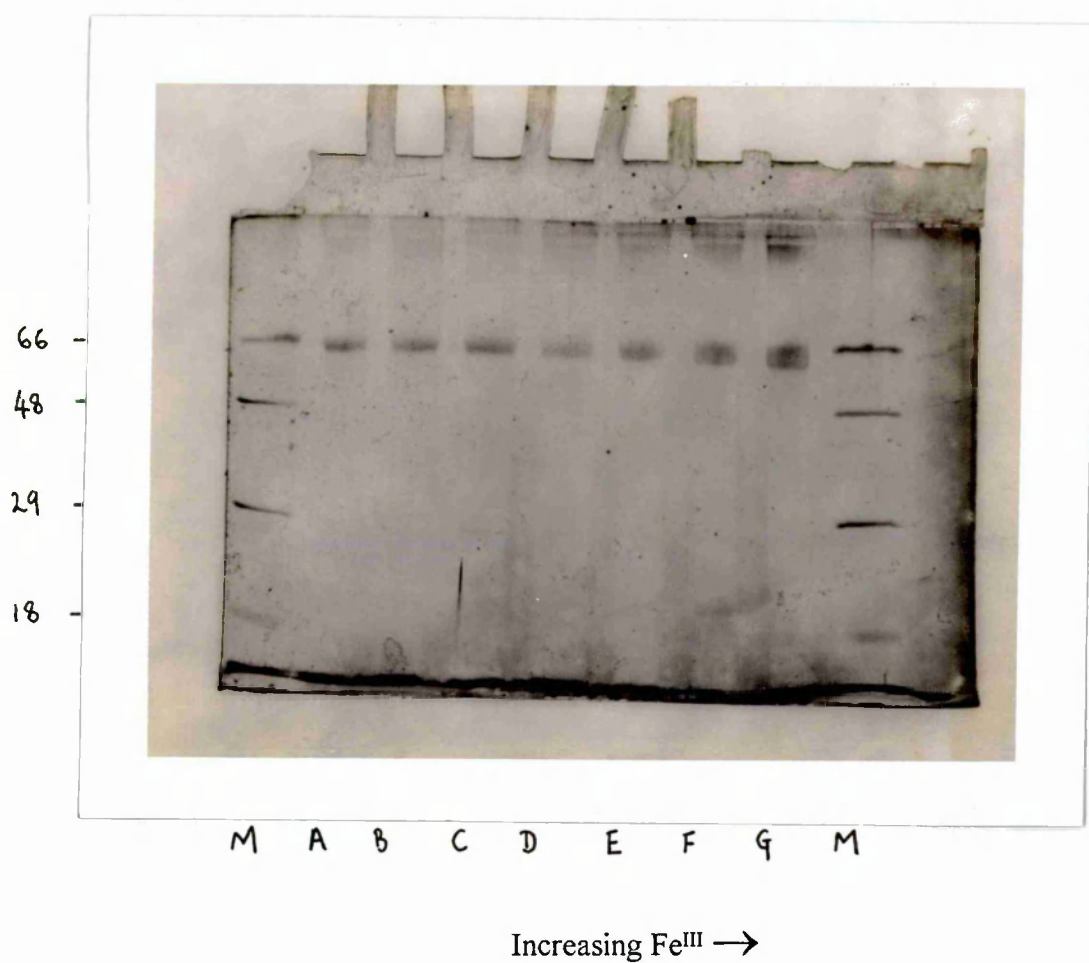


Figure 3.4.12: Fragmentation of native HSA incubated with glucose and increasing concentrations of iron. Lanes A and B are native HSA incubated alone and with 25mM glucose respectively. Lanes C to G have native HSA incubated with glucose and iron chloride at concentrations of 10, 25, 50, 100 and 200 μM respectively.

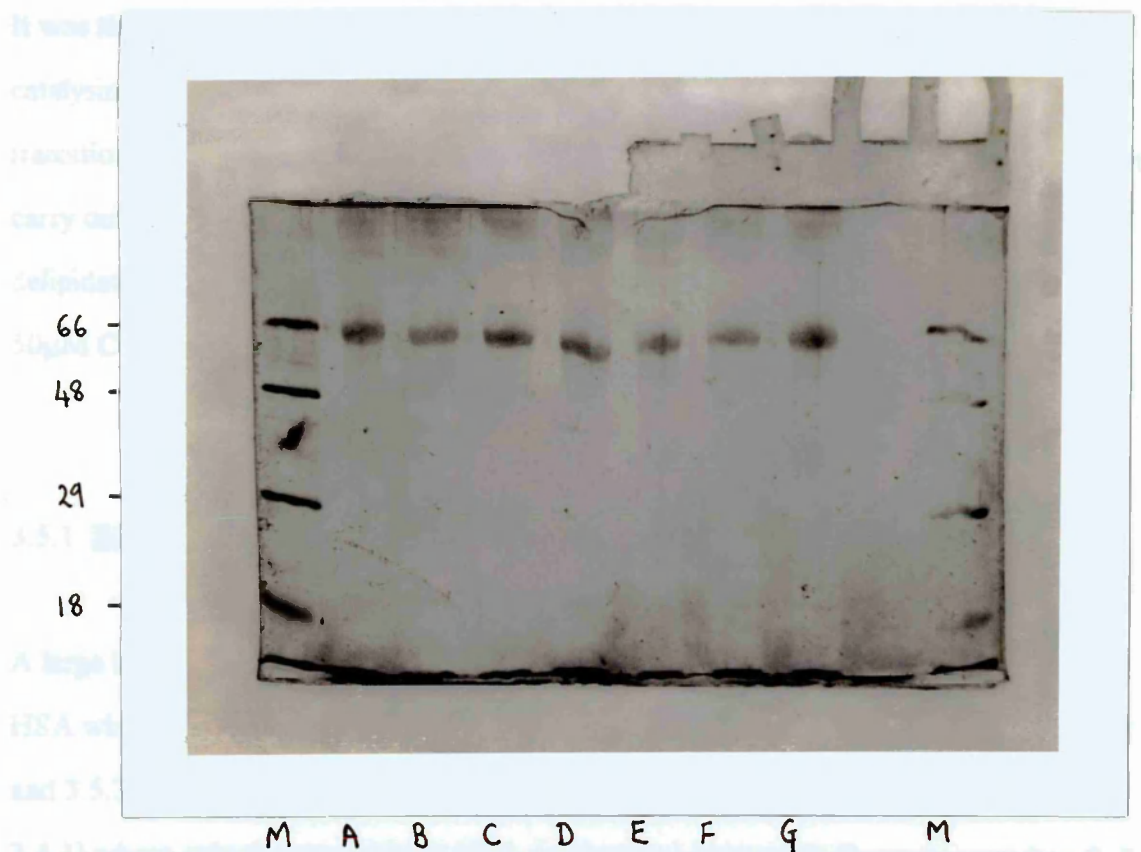


Figure 3.4.13: Fragmentation of delipidated HSA incubated with glucose and increasing concentrations of iron. Lanes A and B are delipidated HSA incubated alone and with 25mM glucose respectively. Lanes C to G have delipidated HSA incubated with glucose and iron chloride at concentrations of 10, 25, 50, 100 and 200 μ M respectively.

3.5 Effect of choice of buffer on AGE formation

It was thought that the phosphate buffer used in earlier work (Figure 3.4.1) was catalysing the production of fluorophores because it contained traces of contaminating transition metals. An alternative low-metal buffer (HEPES, 0.1M, pH 7.4) was chosen to carry out the work shown in Figure 3.5.1. Again the three forms of HSA (native, delipidated and recombinant) were incubated at 10mg/ml with 500mM glucose and 50 μ M CuSO₄ at 37°C for periods of up to 11 days.

3.5.1 Effect of buffer on fluorophore formation

A large increase in glucose induced fluorophore production is seen in all three forms of HSA when Cu^{II} ions are added to the HEPES buffered incubation (Figures 3.5.1, 3.5.2 and 3.5.3). This contrasts with the phosphate buffered incubation (Figures 3.2.2 and 3.4.1) where only the recombinant HSA produces an increase in fluorescence when Cu^{II} is added. In the HEPES buffered system the amount of fluorescence produced with glucose alone is approximately 30% of that when Cu^{II} is also included in each of the incubations. This increase of fluorescence on the addition of Cu^{II} is well in excess of the increase in fluorescence seen in recombinant HSA when Cu^{II} is added to the phosphate buffered system (Figures 3.2.2 and 3.4.1).

The three forms of HSA show similar patterns of fluorescence development in HEPES buffer (Figures 3.5.1, 3.5.2 and 3.5.3), with each of them showing a lag-phase in the development of glycofluorophores over the first 3 to 4 days. The delipidated HSA appears to form similar levels of fluorescence in incubations with either HEPES or sodium phosphate buffers, whilst the two forms of HSA which have bound lipid have a much reduced amount of fluorescence formed when HEPES buffer is used. In Figure 3.5.4 it can be seen that the amount of fluorescence produced by both the native and

recombinant HSAs is similar to that produced by delipidated HSA. Unlike in phosphate buffer, in HEPES buffer there is no enhancement of glycofluorophore production by lipid bound to HSA. It is not possible to compare the fluorescence values from the two experiments directly or precisely as this work was carried out using the Aminco Bowman Spectrofluorimeter which was not able to hold its value for standards from day to day (See Section 2.3.2).

3.5.2 Effect of buffer on protein fragmentation

Fragmentation gels of transferrin incubated with glucose in HEPES buffer and in sodium phosphate buffer (both at 0.1M, pH 7.4) are shown in Figure 3.5.5. Transferrin was incubated at 1mg/ml in each buffer with glucose at 50mM and with copper sulphate added as a source of Cu^{II} ions to the phosphate buffer (100 μM) and to the HEPES buffer at concentrations of 100 μM , 110 μM and 150 μM . The additional CuSO_4 was present in the HEPES to compensate for the traces of contaminating transition metals in the phosphate buffer (approximately 6 μM). Figure 3.5.5 shows that even with enhanced concentrations of Cu^{II} ions beyond the concentration found in phosphate buffer, there is little fragmentation of transferrin in HEPES buffer (lanes D, E and F) compared to the amount of fragmentation that occurs in sodium phosphate buffer (lanes C and J). The fragmentation of transferrin in the phosphate buffered incubation system gives a pattern of fragments similar to that seen in Figure 3.4.6 (lane D). However, transferrin incubated in HEPES buffer produces fewer fragments, with only the fragments of 31 and 42kD appearing. This shows that a small amount of protein fragmentation does occur in HEPES buffered incubations, with the production of both the 16 and 62kD fragments being more fully inhibited.

Figure 3.5.6 shows the fragmentation of native HSA in HEPES buffer. Lane B has a sample of native HSA incubated in the absence of both glucose and Cu^{II} ions. Lanes C to J have samples of native HSA incubated with both glucose and added Cu^{II} ions at various

concentrations. Lane E has a sample of native HSA incubated with 25mM glucose and 100 μ M Cu^{II} which are identical conditions to those in lane D of Figure 3.4.5 except that the buffer used in Figure 3.4.5 is sodium phosphate. Only a very small amount of fragmentation occurs in the HEPES buffer and it does not take the form of a large smear, but 3 to 4 discrete bands with masses between 20 and 26kD. Although some fragmentation of HSA occurs in HEPES buffer, like transferrin, the fragmentation of native HSA can be partially inhibited by the use of HEPES buffer in the place of phosphate buffer.

3.5.3 Summary

These experiments have shown that the use of HEPES buffer in the place of sodium phosphate buffer affects both protein fragmentation and the formation of glycofluorophores. As there is no difference in the amount of fluorophore production on delipidated HSA between the two buffer systems used when Cu^{II} is added, the reduction in the amount of fluorophore produced on native HSA in HEPES is in the component that is related to the lipid bound to HSA. Fragmentation of both transferrin and native HSA is partially inhibited by the use of HEPES buffer, and the pattern of fragments produced in HEPES is different from that in phosphate for each protein. Furthermore, Figure 3.5.5 shows that the enhanced fragmentation of transferrin in phosphate buffer is not due to the trace amounts of transition metal catalysts found in that buffer as even with added amounts of Cu^{II}, well in excess of those found in phosphate buffer, little fragmentation is observed in the HEPES buffered incubations.

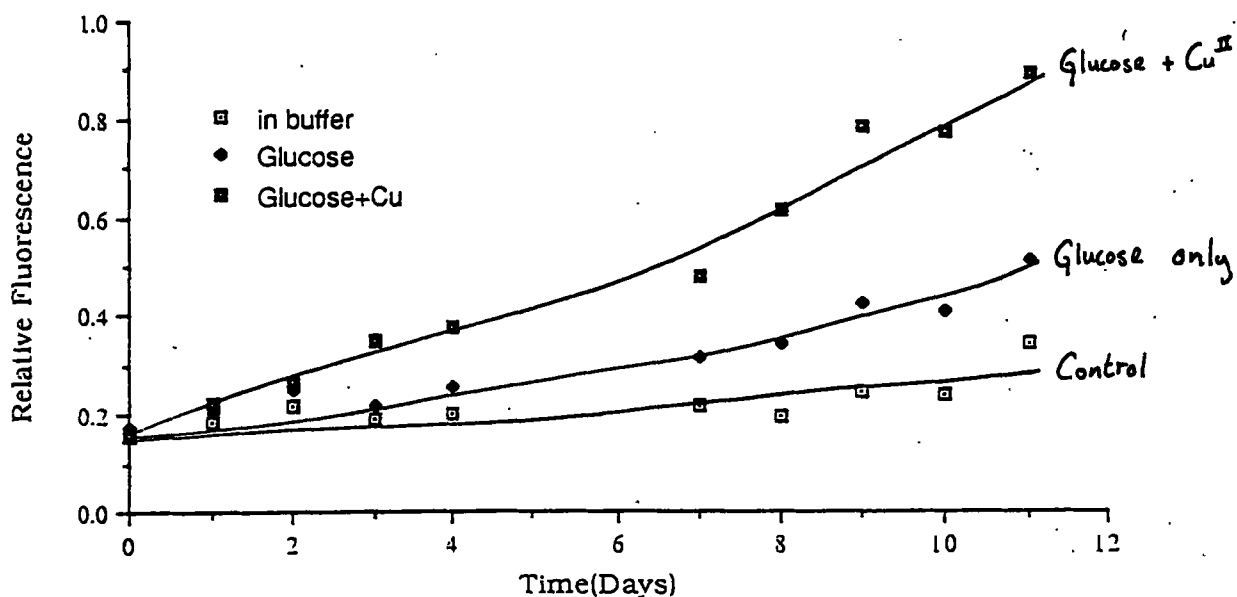


Figure 3.5.1: Fluorescence of native HSA incubated in HEPES.

Fluorescence at excitation 325nm and emission 375nm of native HSA incubated in 0.1M HEPES buffer, pH7.4, at 10mg/ml with glucose (500mM) and CuSO₄ (50μM) at 37°C over a period of up to 11 days.

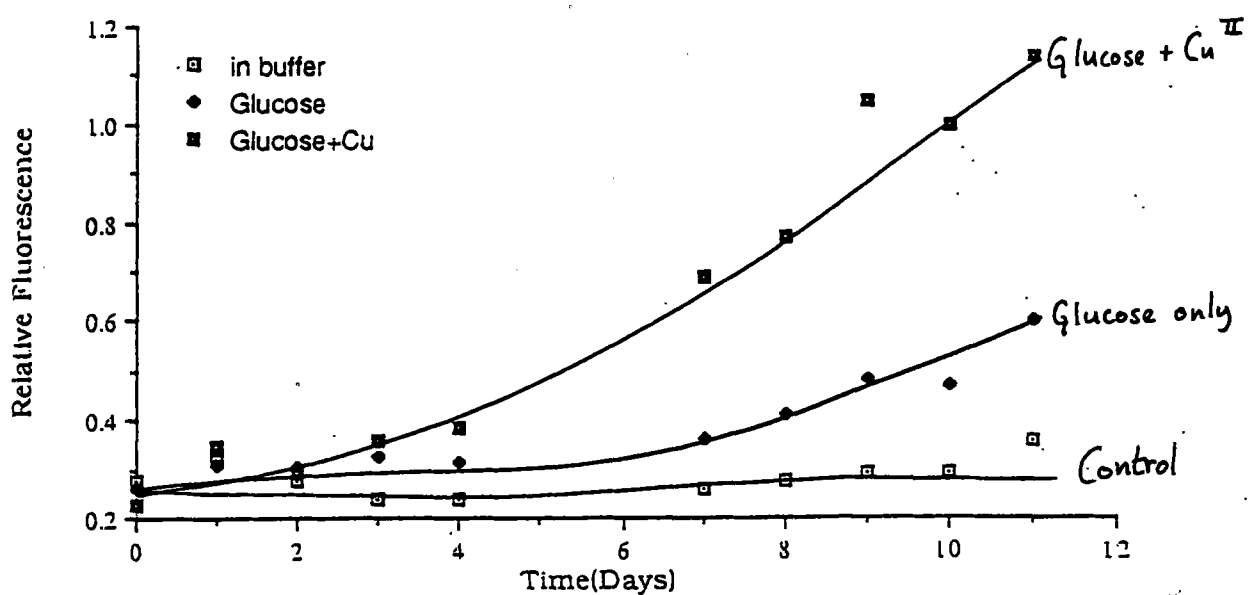


Figure 3.5.2: Fluorescence of delipidated HSA incubated in HEPES.

Fluorescence at excitation 325nm and emission 375nm of delipidated HSA incubated in 0.1M HEPES buffer, pH7.4, at 10mg/ml with glucose (500mM) and CuSO_4 (50 μM) at 37°C over a period of up to 11 days.

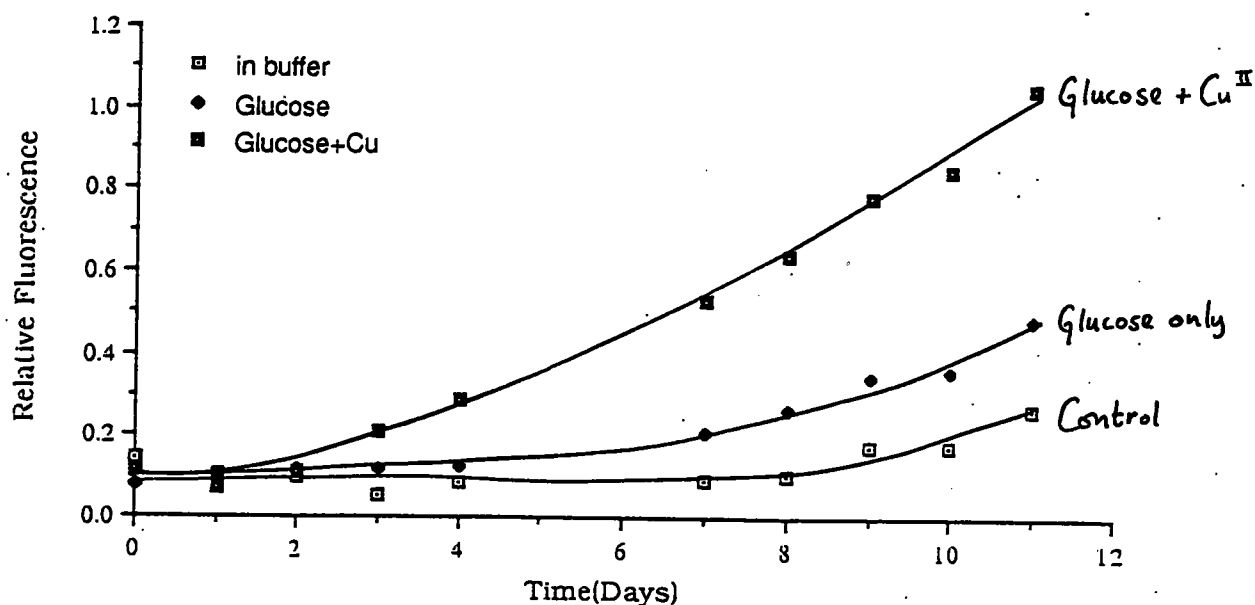


Figure 3.5.3: Fluorescence of recombinant HSA incubated in HEPES.

Fluorescence at excitation 325nm and emission 375nm of recombinant HSA incubated in 0.1M HEPES buffer, pH7.4, at 10mg/ml with glucose (500mM) and CuSO_4 (50 μM) at 37°C over a period of up to 11 days.

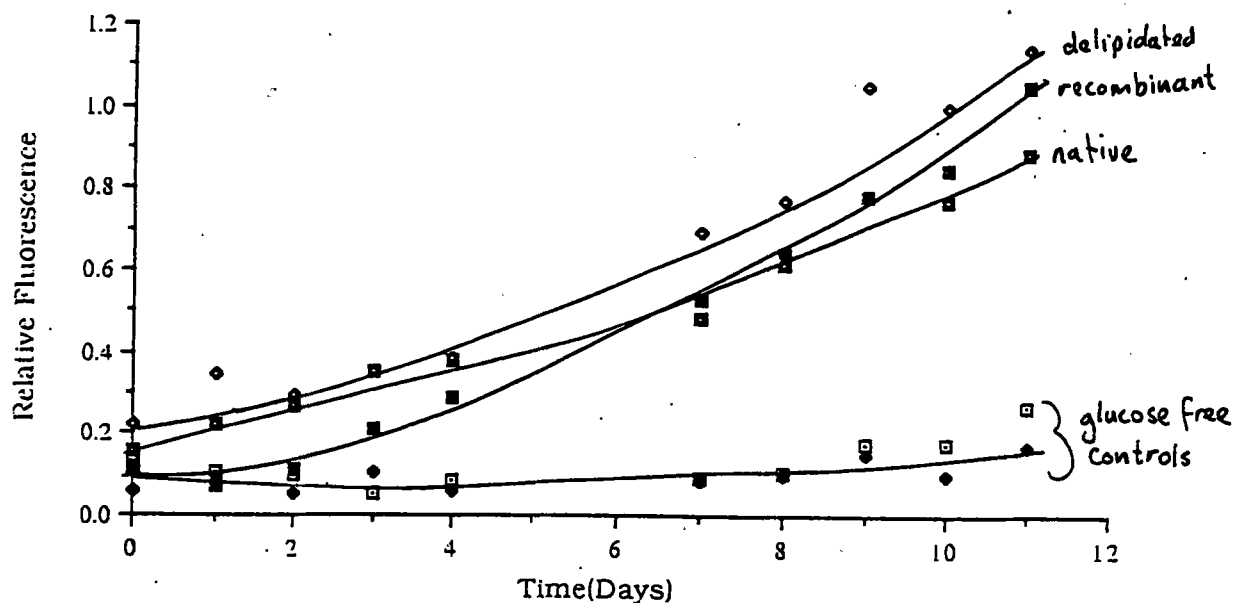


Figure 3.5.4: Fluorescence of native, delipidated and recombinant HSA incubated in HEPES. Comparative fluorescence of native, delipidated and recombinant HSA (10mg/ml) incubated in 0.1M HEPES buffer (pH7.4) with glucose (500mM) and CuSO_4 (50 μM).

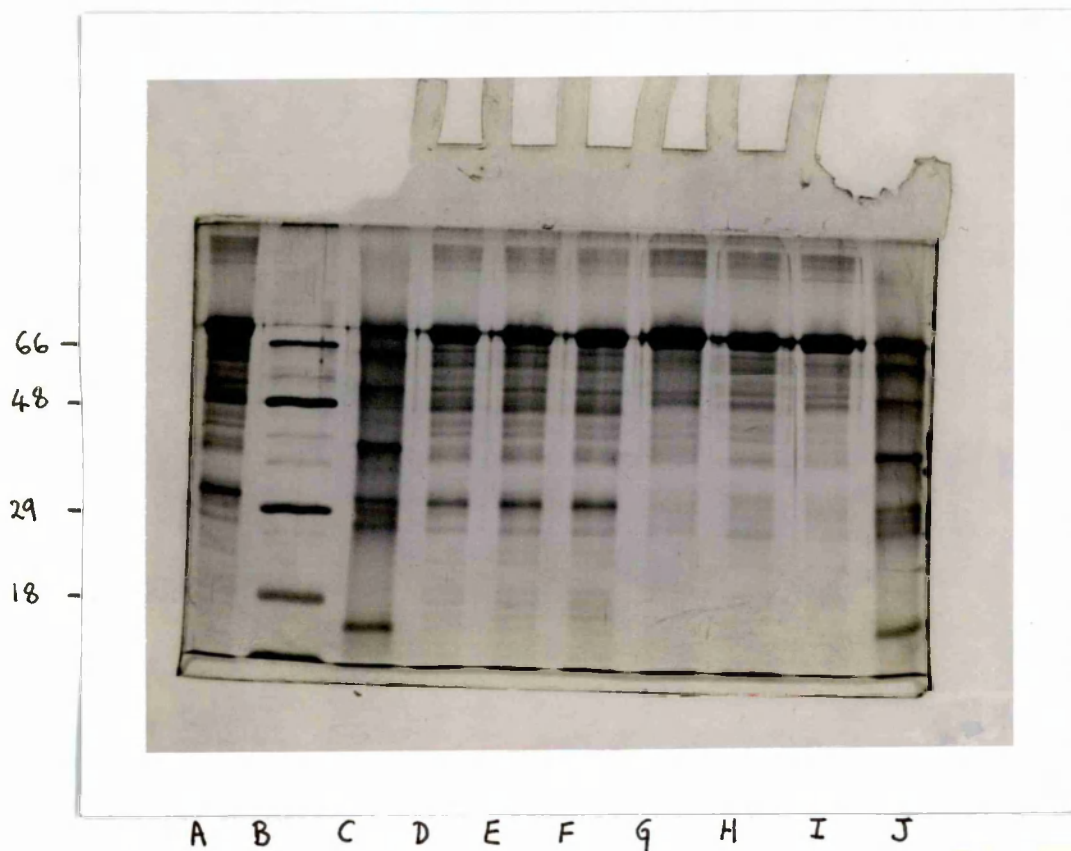


Figure 3.5.5: Fragmentation of transferrin incubated in HEPES or phosphate buffers. 10% gel of transferrin incubated in 0.1M buffer (either HEPES or sodium phosphate) with glucose (50mM) and CuSO_4 for 8 days. Lane C contains protein incubated in phosphate with 100 μM Cu^{II} , Lanes D, E and F are incubations in HEPES with 100, 110 and 150 μM Cu^{II} respectively. Lanes G and H contain transferrin incubated with glucose alone in phosphate and HEPES respectively and Lane I contains transferrin incubated without glucose in phosphate buffer.

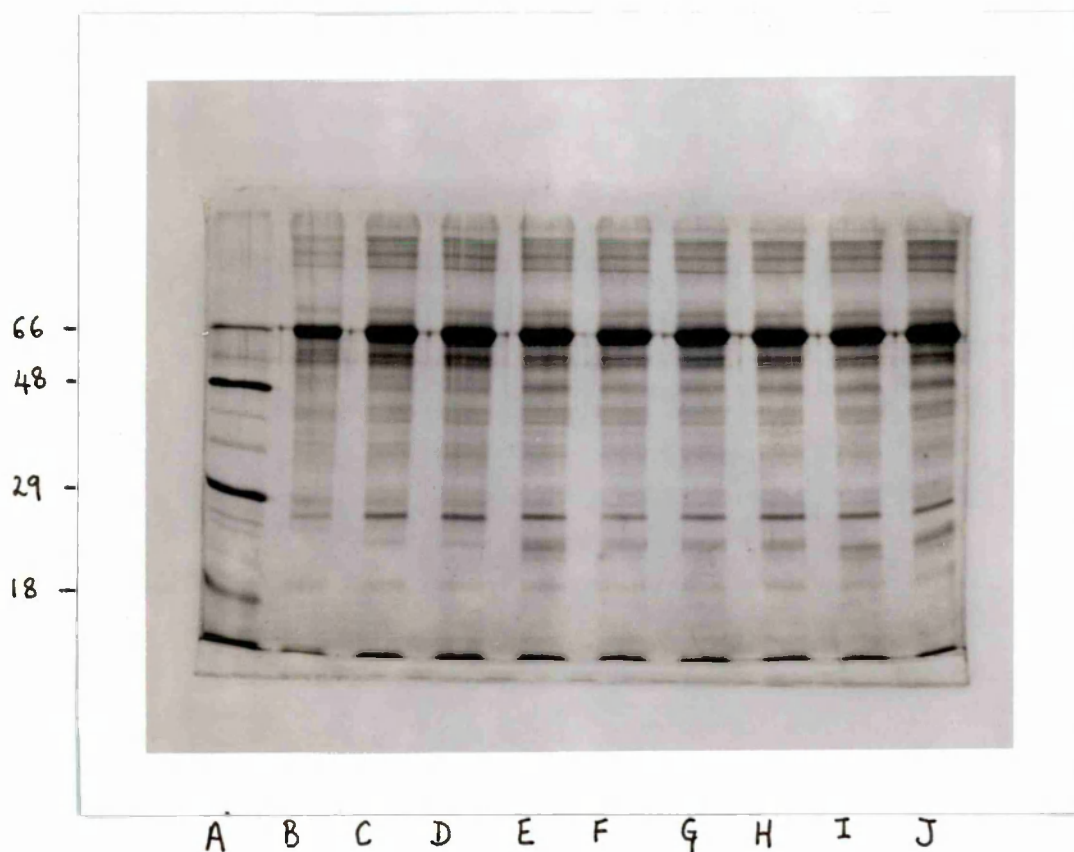


Figure 3.5.6: Fragmentation of native HSA incubated in HEPES.

Native HSA (1mg/ml) was incubated in 0.1M HEPES buffer. Lane B has HSA in buffer alone; Lanes C, D and E have HSA with glucose (25mM) and 25, 50 and 100 μ M CuSO₄ respectively. Lanes F, G and H have HSA with glucose (10mM) and CuSO₄ as above. Lanes I and J have HSA with glucose (20mM) and 50 and 100 μ M CuSO₄ respectively.

3.6 Effect of glucose preincubation

In order to see whether AGE production was affected by glucose undergoing a metal catalysed oxidation reaction prior to contact with the proteins, glucose was incubated in phosphate buffer with and without added Cu^{II} ($10\mu\text{M}$) before incubating it with HSA. In Figure 3.6.2, delipidated HSA incubated with fresh glucose shows a lag-phase of about 2 days before fluorophore production begins, whether the incubation media has added Cu^{II} ions or not. The samples of delipidated HSA with preincubated glucose do not show this same lag-phase in fluorophore production and clearly have faster initial rates. A slight enhancement of the initial rate of fluorophore production in recombinant HSA (Figure 3.6.3) is seen when the glucose has been preincubated. The most noticeable feature in this figure is the difference in the rate of fluorophore formation between the samples incubated with and without Cu^{II} in the final incubation regardless of whether the glucose was preincubated.

Fluorescence formation in native HSA (Figure 3.6.1), which unlike that of recombinant HSA is not enhanced by the presence of additional Cu^{II} in the incubation (Figures 3.2.2 and 3.4.1) does not have the lag-phase when the protein is incubated in phosphate buffer with fresh glucose. No enhancement in the initial rate of fluorophore production is seen in native HSA when the glucose used in the incubation has been preincubated in phosphate buffer either with or without additional Cu^{II} ions.

It can be seen that the preincubation of glucose influences the production of fluorescent AGEs in both delipidated and recombinant HSA to at least a small extent. From this data it is not possible to tell if the oxidation of glucose prior to reaction with protein is only a minor side reaction contributing only to the initial enhancement of glycofluorophore production which covers the period of the lag-phase or if the lag-phase seen with fresh glucose is the result of the time taken for the products of glucose autoxidation to build up.

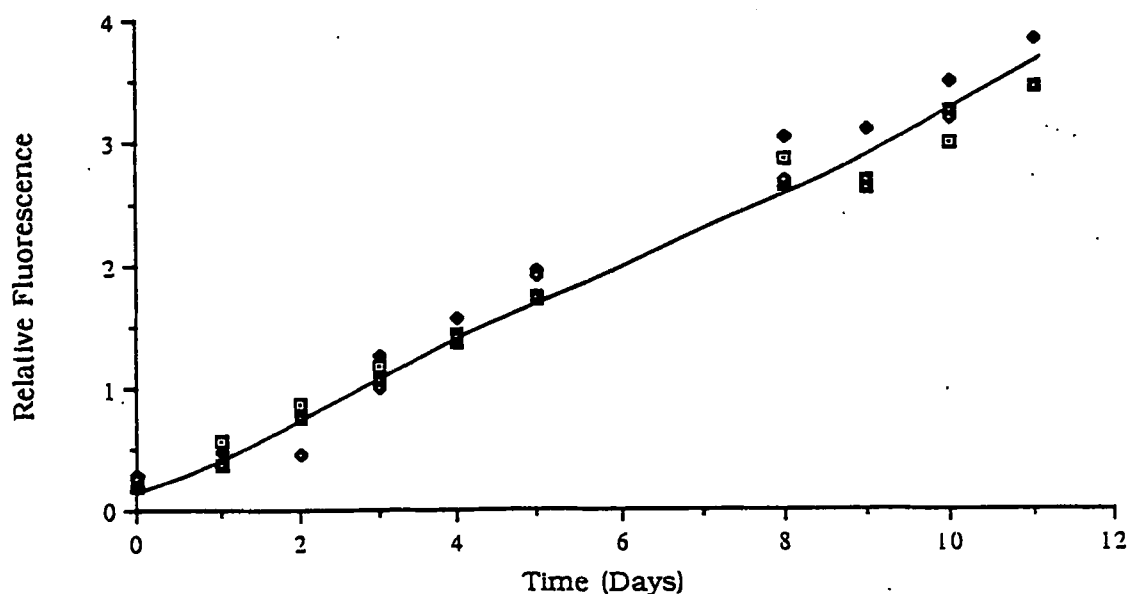


Figure 3.6.1: Fluorescence of native HSA incubated with copper and preincubated glucose. Native HSA incubated at 10mg/ml in 0.1M sodium phosphate buffer (pH7.4) at 37°C for up to 11 days. Glucose was included in all samples at 500mM. Two of the samples were incubated with fresh glucose, one with added CuSO_4 (1 μM), (■) and one without (◆). In addition to this, two of the samples of glucose had been pre-incubated in phosphate buffer for 3 days, one with CuSO_4 (10 μM) in the pre-incubation medium (■), and the other having copper added to it when the incubation with protein began (◆).

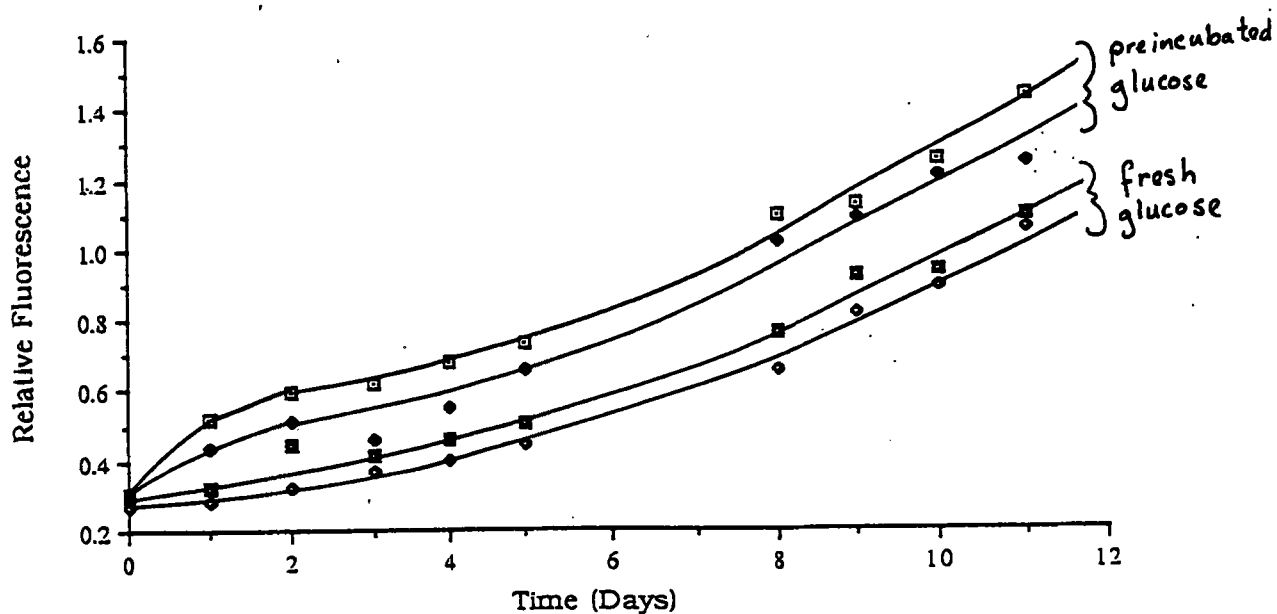


Figure 3.6.2: Fluorescence of delipidated HSA incubated with copper and preincubated glucose. Delipidated HSA incubated at 10mg/ml in 0.1M sodium phosphate buffer (pH7.4) at 37°C for up to 11 days. Glucose was included in all samples at 500mM. Two of the samples were incubated with fresh glucose, one with added CuSO₄ (1μM), (■) and one without (◆). In addition to this, two of the samples of glucose had been pre-incubated in phosphate buffer for 3 days, one with CuSO₄ (10μM) in the pre-incubation medium (■), and the other having copper added to it when the incubation with protein began (◆).

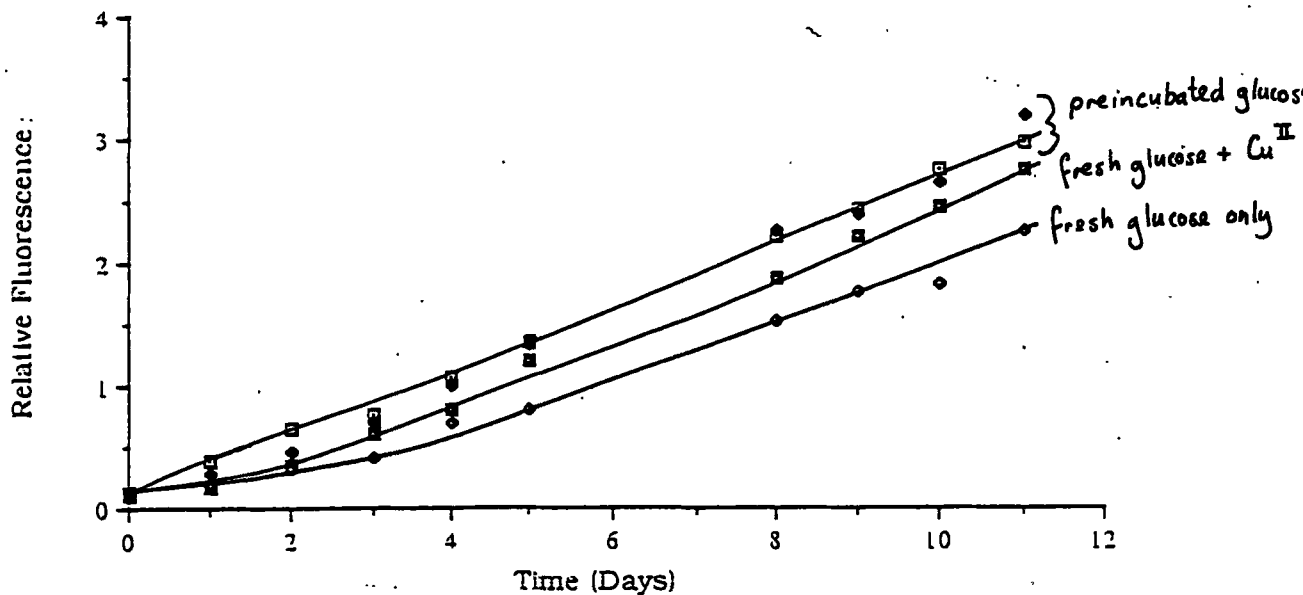


Figure 3.6.3: Fluorescence of recombinant HSA incubated with copper and preincubated glucose. Recombinant HSA incubated at 10mg/ml in 0.1M sodium phosphate buffer (pH7.4) at 37°C for up to 11 days. Glucose was included in all samples at 500mM. Two of the samples were incubated with fresh glucose, one with added CuSO₄ (1μM), (■) and one without (●). In addition to this, two of the samples of glucose had been pre-incubated in phosphate buffer for 3 days, one with CuSO₄ (10μM) in the pre-incubation medium (□), and the other having copper added to it when the incubation with protein began (●).

3.7 Behaviour of different reducing sugars

Sugar-induced changes to protein structure are known to occur at different rates for different sugars. Furthermore, a different picture is seen if the reaction is monitored at an early stage, i.e. the formation of Amadori product, or at a later stage, e.g. the formation of glycofluorophores. I therefore compared the amounts of Amadori product and glycofluorophores produced in a set time when HSA was incubated with glucose or fructose. Native HSA was incubated for 5 days in 0.05M sodium phosphate buffer (pH7.4 / 3mM sodium azide), at 37°C with 10mM and 500mM glucose or 10mM fructose.

3.7.1 Production of Amadori product by both glucose and fructose

The samples were extensively dialysed to remove unbound sugar and the protein and periodate assays were carried out to establish the number of moles of Amadori product per mole of protein. There was little difference in the amount of Amadori product detected by the periodate assay for the protein incubated with either 10mM glucose or 10mM fructose. The protein incubated with 500mM glucose showed a large increase in the number of moles of Amadori product per mole of protein (Figure 3.7.1)

3.7.2 Production of glycofluorophores by glucose and fructose

Fluorescence was measured at both excitation 325nm/emission 375nm and excitation 370nm/emission 440nm. Figures 3.7.2 and 3.7.3 show fluorophore production at both sets of wavelengths respectively. Little or no fluorophore production was seen in the sample incubated in 10mM glucose. The sample incubated with 500mM glucose showed fluorophore production which was similar at both the wavelengths. The largest increase

in fluorophore production was seen in the samples which were incubated with 10mM fructose. Unlike the incubations in glucose, in which the fluorescence at both wavelengths was very similar, the fluorescence produced at excitation 370nm and emission 440nm was markedly greater than that produced at the other wavelength.

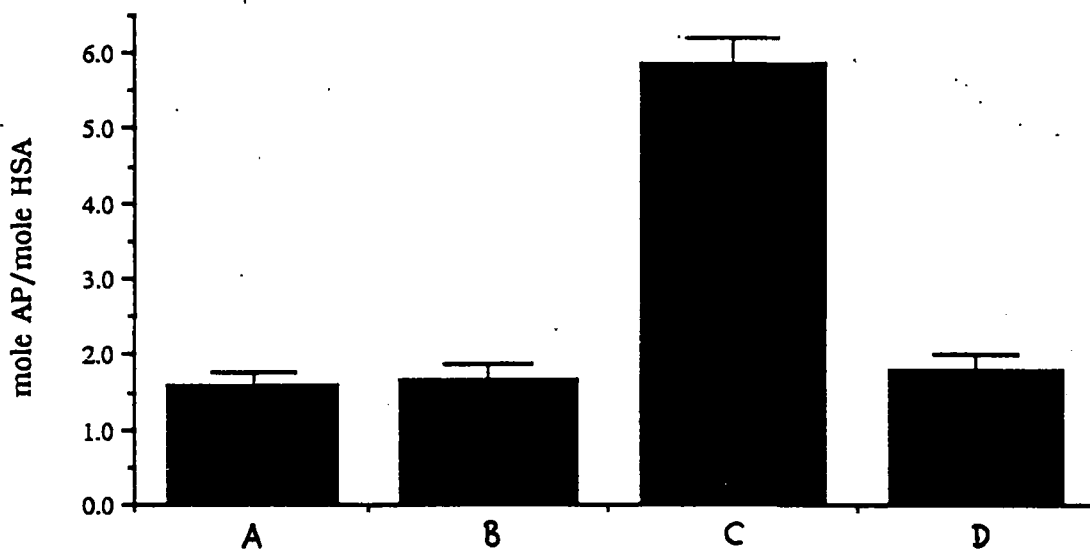


Figure 3.7.1: Formation of Amadori product on native HSA incubated with either fructose or glucose. Amount of Amadori product formed per mole of protein when native HSA (10mg/ml) was incubated with 10mM and 500mM glucose and 10mM fructose in 0.05M sodium phosphate buffer (pH 7.4) at 37°C for 5 days. (A) represents incubation without sugars, (B) incubation with 10mM glucose, (C) incubation with 500mM glucose and (D) incubation 10mM fructose. Error bars show 1 standard deviation, where $n=3$.

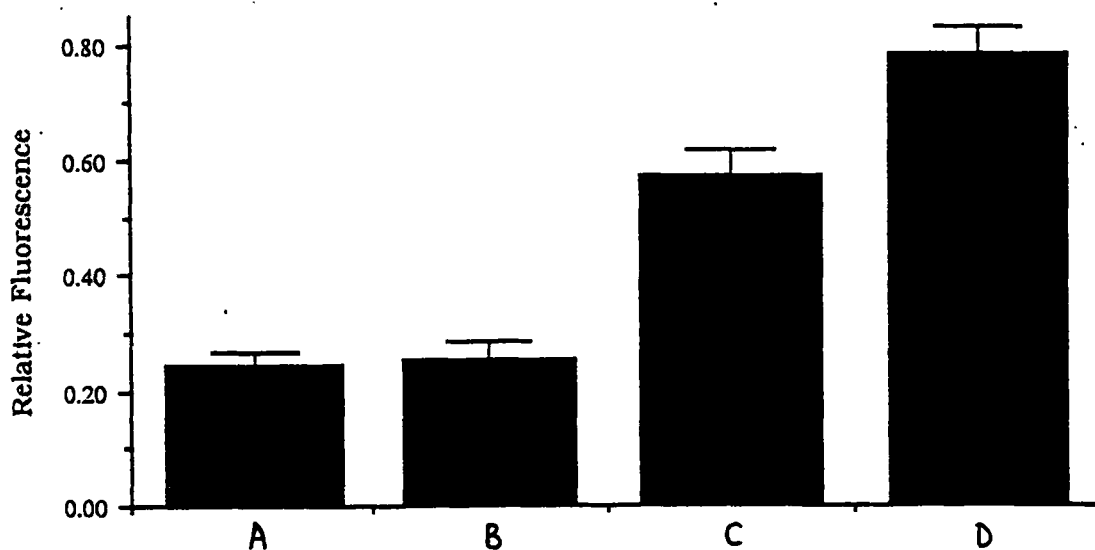


Figure 3.7.2: Fluorescence of native HSA incubated with glucose or fructose. Fluorescence at excitation 325nm and emission 375nm of native HSA (10mg/ml) incubated with 10mM and 500mM glucose and 10mM fructose in 0.05M sodium phosphate buffer (pH 7.4) at 37°C for 5 days. (A) represents incubation without sugars, (B) incubation with 10mM glucose, (C) incubation with 500mM glucose and (D) incubation 10mM fructose. Error bars show 1 standard deviation, where n=3.

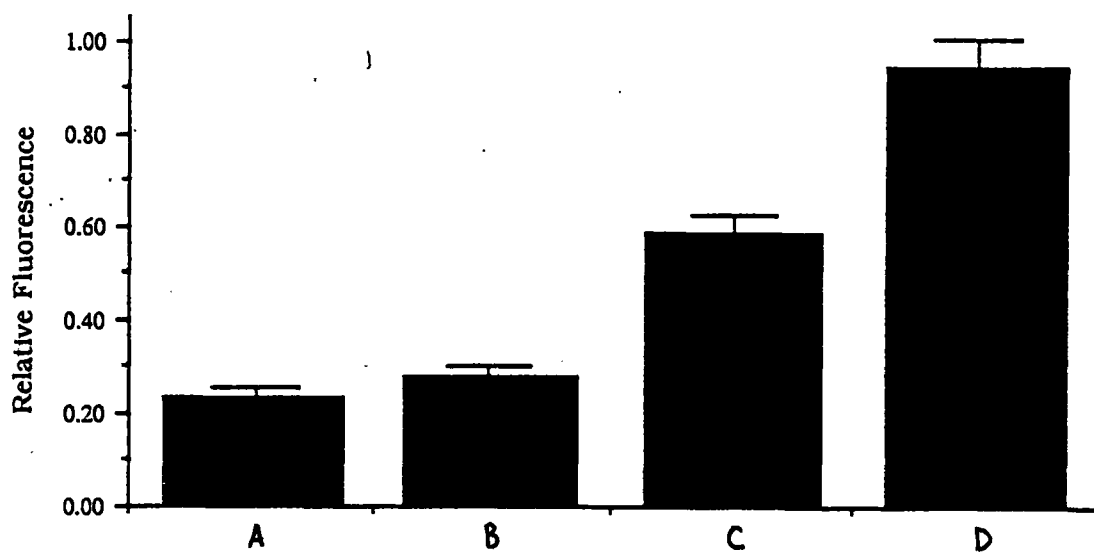


Figure 3.7.3: Fluorescence at excitation 370nm and emission 440nm of native HSA incubated with glucose and fructose. Fluorescence at excitation 370nm and emission 440nm of native HSA (10mg/ml) incubated with 10mM and 500mM glucose and 10mM fructose in 0.05M sodium phosphate buffer (pH 7.4) at 37°C for 5 days. (A) represents incubation without sugars, (B) incubation with 10mM glucose, (C) incubation with 500mM glucose and (D) incubation 10mM fructose. Error bars show 1 standard deviation, where $n=3$.

3.8 Inhibition of AGE formation

Four compounds were looked at with respect to their abilities to inhibit the reactions that produce fluorophores. Transferrin and native and delipidated HSA were incubated at 2 mg/ml in 0.1M sodium phosphate buffer, pH 7.4, with glucose (100mM) for 8 days at 37°C. Also included in the incubations were the compounds aminoguanidine, propyl gallate, trolox (a water soluble vitamin E derivative) and acetyl salicylic acid, each at concentrations of 25µM, 250µM, 2.5mM, 10mM and 25mM. Fluorescence was measured at excitation 325nm and emission 375nm.

3.8.1 Aminoguanidine

Aminoguanidine is thought to block glycofluorophore formation by scavenging dicarbonyl compounds that are formed either from the breakdown of Amadori product or directly from fructose. It is thought that these dicarbonyl compounds play a major role in the formation of glycofluorophores. Figure 3.8.1 shows the fluorescence of native and delipidated HSA and transferrin where aminoguanidine is being used as an inhibitor.

Aminoguanidine incubated with all three proteins in the absence of glucose gives a very similar fluorescence value to that of the proteins incubated in buffer alone. The inhibition of protein fluorescence in the presence of glucose is dose dependent with respect to the concentration of aminoguanidine in the incubation mixture. Fluorescence formation at 25µM aminoguanidine is approximately 50% of the uninhibited fluorescence formation for both forms of HSA, but transferrin appears to need much higher concentrations of aminoguanidine to inhibit fluorescence formation to the same extent.

3.8.2 Propyl Gallate

Propyl gallate is a free radical scavenger which also has transition metal binding properties. Figure 3.8.2 shows the fluorescence production for native and delipidated HSA and transferrin where the compound propyl gallate is being tested as an inhibitor. The fluorescence of propyl gallate incubated alone with protein is significantly higher than that of protein incubated alone, although it is still much lower than the values obtained for each of the three proteins incubated with glucose. Propyl gallate inhibits fluorescence formation in all three proteins even at 25 μ M. Inhibition of fluorescence at this concentration of propyl gallate is much greater in native HSA than in transferrin, with inhibition in delipidated HSA falling somewhere between the other two proteins. High concentrations of propyl gallate in the presence of glucose produce large amounts of fluorescence and in the case of HSA both proteins produce greater amounts of fluorescence with 25mM propyl gallate when glucose is present than when glucose is absent from the incubation. It seems that inhibition of glucose derived fluorescence is either being masked by fluorescence derived from an interaction between propyl gallate and glucose or that inhibition is no longer occurring at higher concentrations of propyl gallate.

3.8.3 Trolox

Vitamin E is a well known scavenger of free radicals and trolox is a water soluble derivative of this compound. Figure 3.8.3 shows the effect of trolox on fluorescence production in native and delipidated HSA and transferrin incubated with glucose. The control sample containing trolox incubated alone with each protein has a very high fluorescence value, which in delipidated HSA is nearly as high as the fluorescence produced in the control containing the protein with glucose. In transferrin the fluorescence produced by incubation of trolox alone with transferrin is very much greater than that produced by transferrin incubated with glucose alone. Inhibition of glucose induced fluorescence is seen in both native and delipidated HSA although at high

concentrations of trolox this effect is masked by the amount of fluorescence produced by the increasing concentrations of trolox. No inhibition of glucose derived fluorescence is seen in transferrin incubated even with very low concentrations of trolox. The fluorescence of these samples rises in a dose-dependent manner with the concentration of trolox.

3.8.4 Acetyl Salicyclic Acid

The fluorescence of all three proteins incubated with acetyl salicyclic acid is much greater than the fluorescence obtained from samples where the proteins were incubated with glucose alone. This difference in fluorescence values is so great that the samples where proteins were incubated with glucose alone barely show on the same scale of graph as those incubated with acetyl salicyclic acid either in the presence or absence of glucose. Little information on whether there is any inhibition of glycofluorophore production can be obtained from these results, as at present there is no way of separating the the fluorscence derived from the acetyl salicyclic acid from the glucose derived fluorescence.

3.8.5 Summary

Aminoguanidine is a good inhibitor of fluorophore production as monitored at excitation 325nm and emission 375nm. Both propyl gallate and trolox act as inhibitors of this reaction (except for fluorophore production on transferrin in the presence of trolox). In all three proteins high concentrations of both propyl gallate and trolox give misleading results as some new fluorophore is produced which masks the formation (or inhibition) of glucose derived fluorophores on the three proteins. With both these compounds, production of a yellow chromophore is dependent on the concentration of the inhibitor and not on the presence of glucose in the incubation media. Acetyl salicyclic acid is a very effective promoter of fluorescence at the wavelengths used here and due to this it is impossible to see if it inhibits the formation of glucose induced fluorophores.

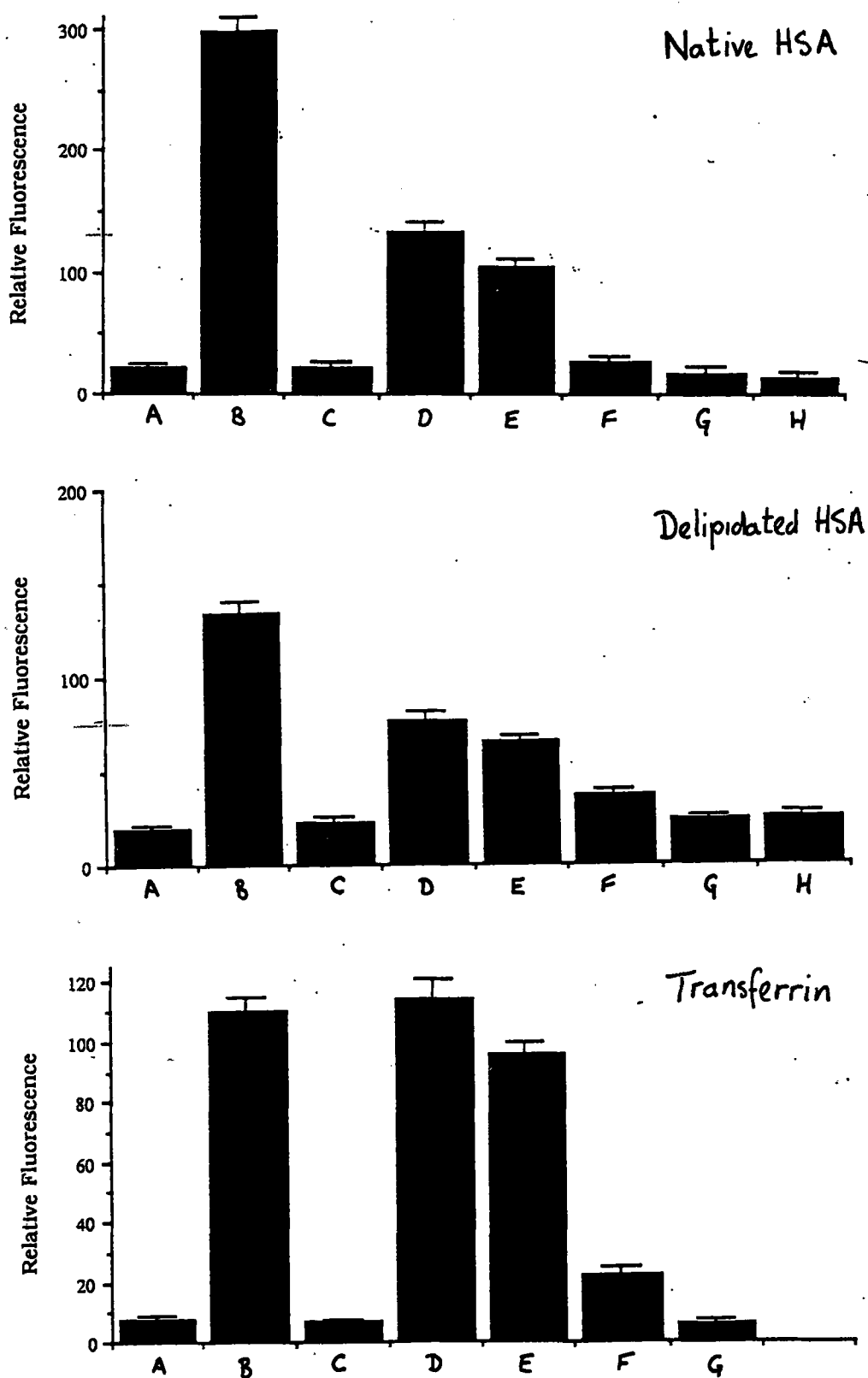


Figure 3.8.1: Aminoguanidine as an inhibitor

Fluorescence of native and delipidated HSA and transferrin incubated in phosphate buffer only (A), with 100mM glucose (B), with 25mM aminoguanidine (C) and with 100mM glucose and aminoguanidine at 25 μ M (D), 250 μ M (E), 2.5mM (F), 10mM (G) and 25mM (H).

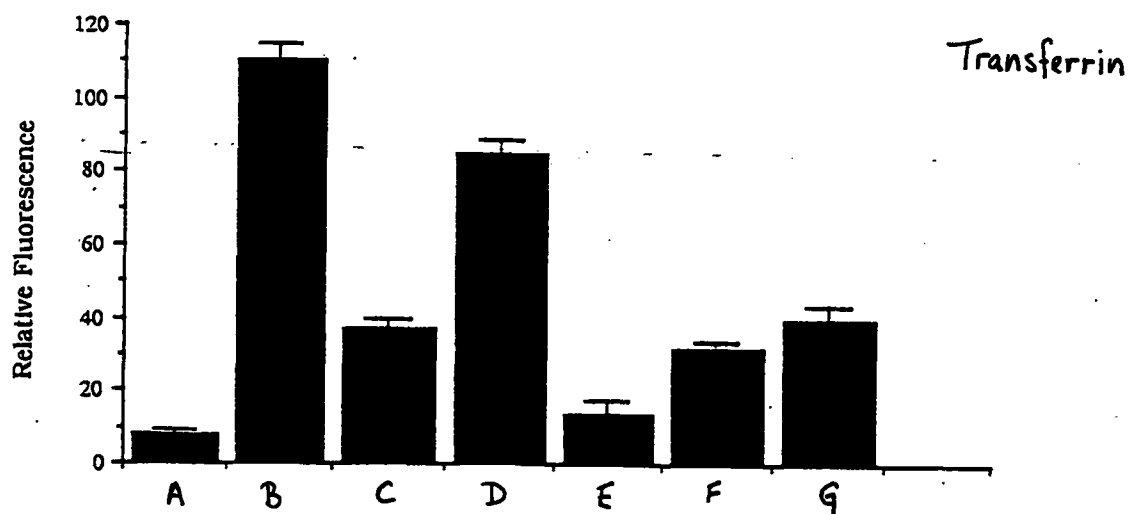
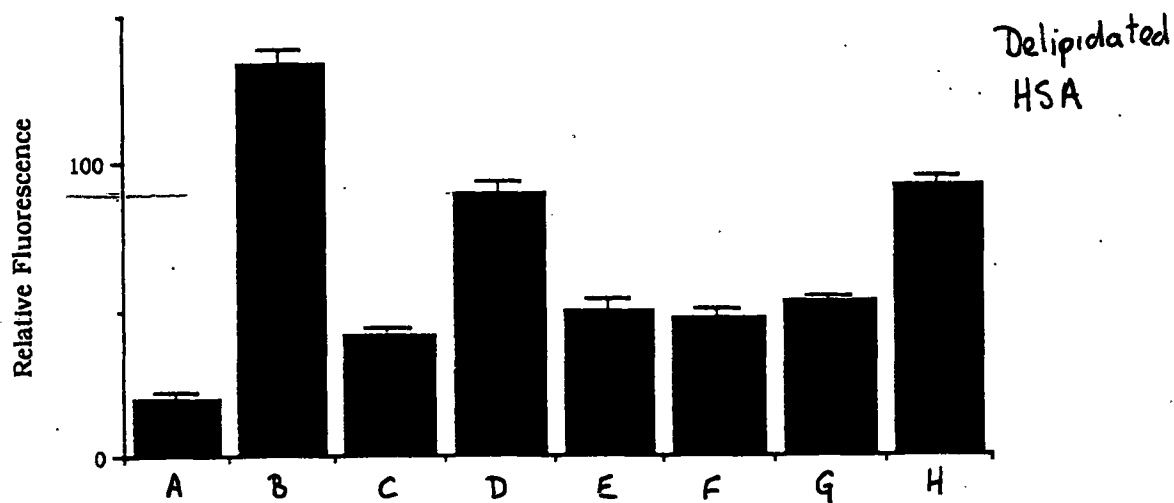
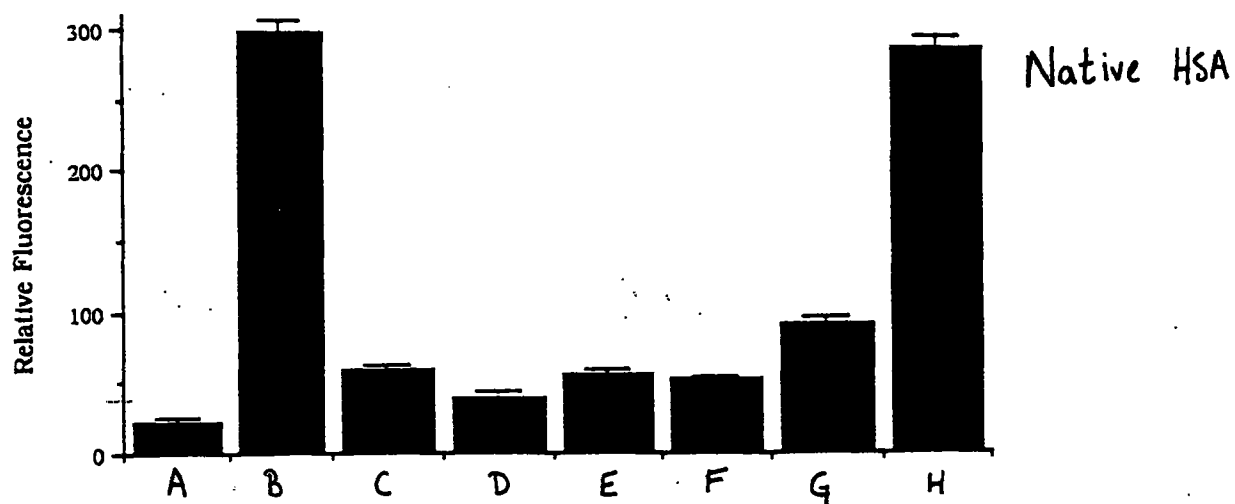


Figure 3.8.2: Propyl gallate as an inhibitor

Fluorescence of native and delipidated HSA and transferrin incubated in phosphate buffer only (A), with 100mM glucose (B), with 25mM propyl gallate (C) and with 100mM glucose and propyl gallate at 25 μ M (D), 250 μ M (E), 2.5mM (F), 10mM (G) and 25mM (H).

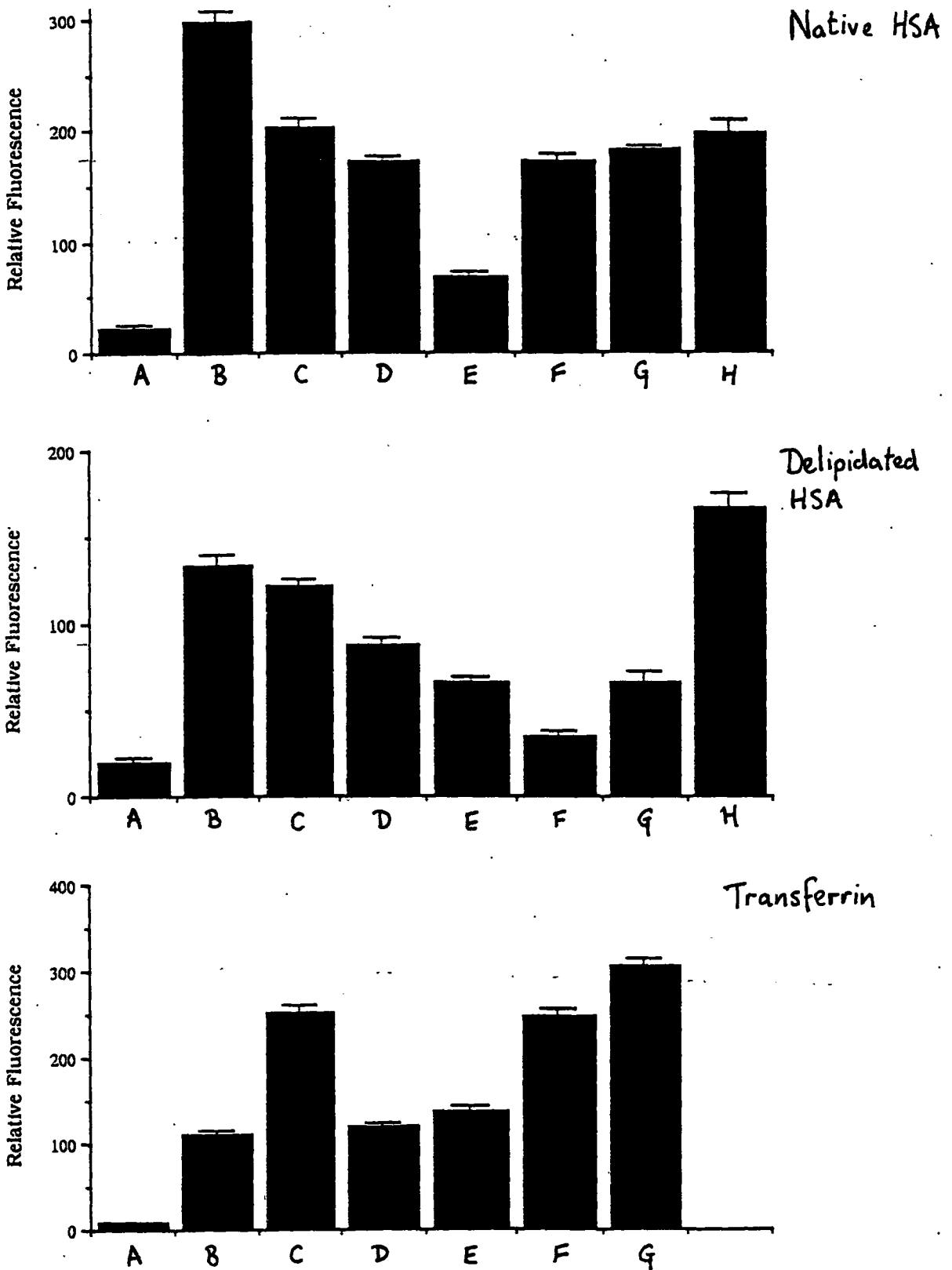


Figure 3.8.3: Trolox as an inhibitor

Fluorescence of native and delipidated HSA and transferrin incubated in phosphate buffer only (A), with 100mM glucose (B), with 25mM trolox (C) and with 100mM glucose and trolox at 25 μ M (D), 250 μ M (E), 2.5mM (F), 10mM (G) and 25mM (H).

3.9 Effect of relative concentrations of protein and glucose

It is known that the formation of Amadori product is effectively first order with respect to glucose concentration providing that other conditions are kept constant (see Figure 3.2.1). Throughout the literature (and also this project) different concentrations of proteins and sugars are used for *in vitro* investigations. Therefore the following experiment was carried out in an attempt to determine whether fluorescent AGEs are dependent on the relative concentrations of glucose and protein in the same way as the formation of the Amadori product.

Transferrin was incubated at 1, 10, 20 and 40 mg/ml in 0.1M sodium phosphate buffer, pH 7.4, for 8 days and the fluorescence of these samples was measured at excitation 325nm and emission 375nm. Samples included glucose at concentrations of 25 or 100mM, and CuSO₄ at 100μM.

Figure 3.9.1 shows the increase in fluorescence (compared to the fluorescence of transferrin incubated without glucose) of transferrin incubated at 1, 10, 20 and 40 mg/ml under the four different sets of conditions. All samples of transferrin were diluted to 0.5mg/ml before fluorescence was measured. For every set of conditions used the fluorescence produced by the sample of transferrin incubated at 1mg/ml was much greater than for the others. There was a steady reduction of fluorophore formation in parallel with the increasing concentration of protein in the original incubation. The relative molar ratio of glucose to protein was 40 times greater for the sample incubated at 1mg/ml than for the sample incubated at a protein concentration of 40mg/ml, but the observed rise in fluorescence was approximately only 7 fold.

Tables 3.9.1 and 3.9.2 show the molar ratio of both glucose and acyclic glucose to transferrin for all samples and the increase in fluorescence that is observed for each. The same relative concentration of reactants exists when the protein concentration is 40mg/ml

and the glucose concentration is 100mM as when the protein is incubated at 10 mg/ml with 25mM glucose. However, the amount of fluorescence produced by the sample of protein incubated at 40 mg/ml is ten times higher than that produced by the incubation at 10mg/ml. These results show that what appears to be an excess of glucose in *in vitro* reactions, may in fact not be.

Table 3.9.1: Molar ratios of glucose:transferrin and increase in fluorescence when transferrin is incubated with 25mM glucose

Transferrin Concentration	Glucose:Tfn (molar ratio)	Acyclic Glucose:Tfn (molar ratio)	Fluorescence Increase (Arbitrary units)
1mg/ml	1984.1	0.0397	34.9
10 mg/ml	198.1	3.967×10^{-3}	2.2
20 mg/ml	99.2	1.984×10^{-3}	5.7
40 mg/ml	49.6	9.921×10^{-4}	3.3

Table 3.9.2: Molar ratios of glucose:transferrin and increase in fluorescence when transferrin is incubated with 100mM glucose

Transferrin Concentration	Glucose:Tfn (molar ratio)	Acyclic Glucose:Tfn (molar ratio)	Fluorescence Increase (Arbitrary units)
1mg/ml	7936	0.159	169.1
10 mg/ml	793.6	0.0159	42.9
20 mg/ml	396.8	7.936×10^{-3}	31.2
40 mg/ml	198.4	3.97×10^{-3}	20.7

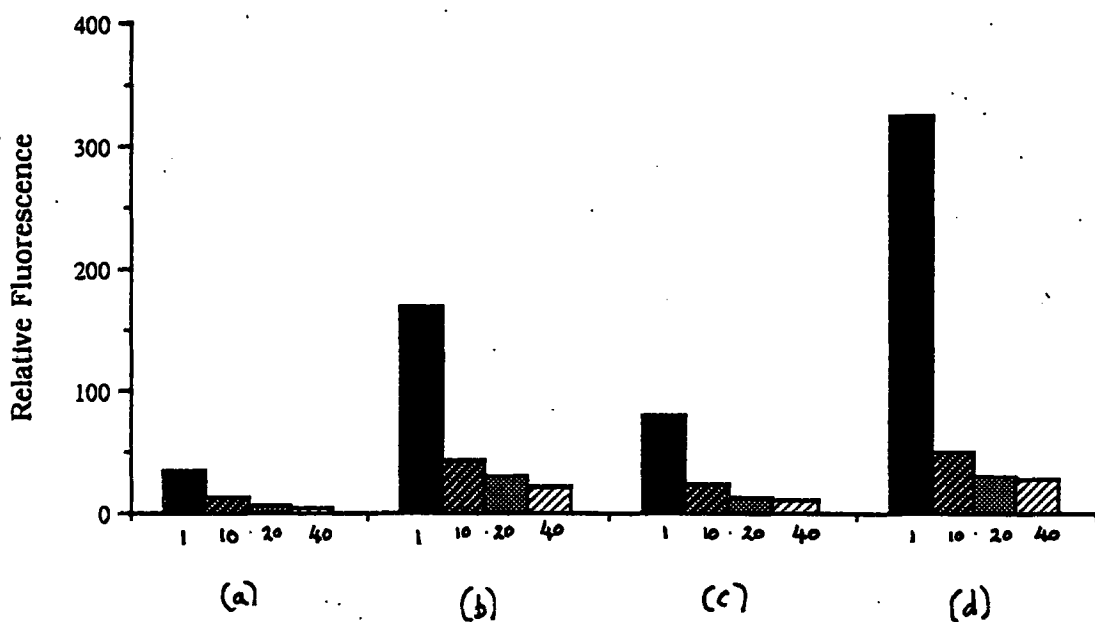


Figure 3.9.1: Fluorescence of transferrin incubated at varying concentrations in both 25mM and 100mM glucose. Fluorescence at excitation 325nm and emission 375nm of transferrin incubated at 1, 10, 20 and 40mg/ml with: (a) 25mM glucose, (b) 100mM glucose, (c) 25mM glucose and 100µM CuSO₄ and (d) 100mM glucose and 100µM CuSO₄.

3.10 Choice of fluorescence wavelengths

Throughout the literature the development of glucose induced fluorophores has been followed at different excitation and emission wavelengths. The three principal sets of fluorescence wavelengths used by other researchers are excitation 325nm/emission 375nm, excitation 370nm/emission 440nm and excitation 360/emission 454nm. Clearly it was impractical to measure the fluorescence in every experiment at all three sets of wavelengths. Therefore, emission scans of both glycated and non-glycated native HSA were carried out at excitation wavelengths of 325, 370 and 360nm in order to decide which set of wavelengths to use. In all three cases the amount of fluorescence produced by the glycated HSA far exceeds the amount produced by the non-glycated HSA (Figure 3.10.1). None of the emission scans show their maxima at the wavelengths listed above. The emission maxima for excitation at 325nm was 405nm, for excitation at 370nm it was 432nm and for excitation at 360 it was 425nm. The increase in fluorescence formation seen on incubation of native HSA with glucose in relation to the fluorescence of the starting material is greatest when the excitation wavelength is 325nm. Therefore the use of 325nm as the exciting wavelength seems to be a good choice for following glucose induced changes in protein fluorescence. Emissions had to be read at 375nm throughout all the experiments so that results obtained could be compared directly with those in the literature.

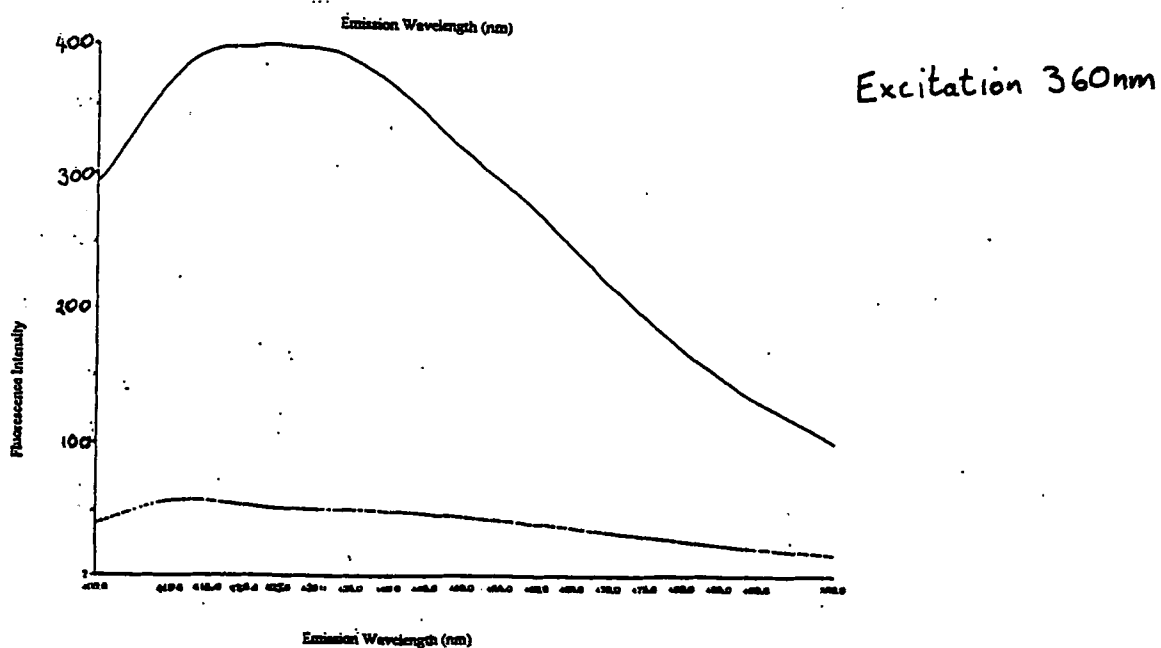
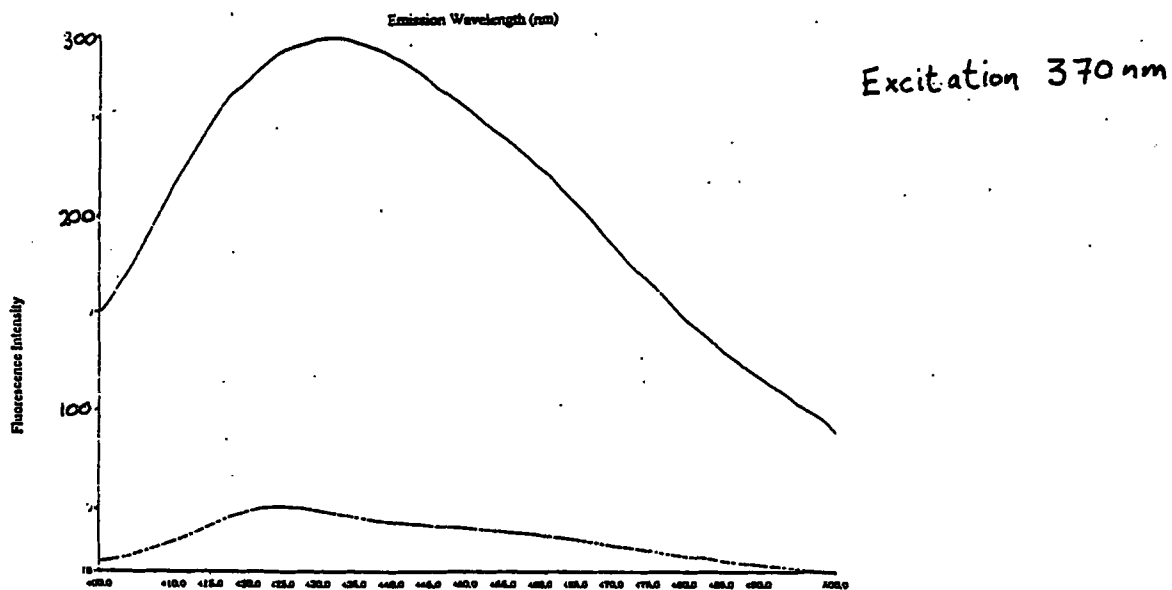
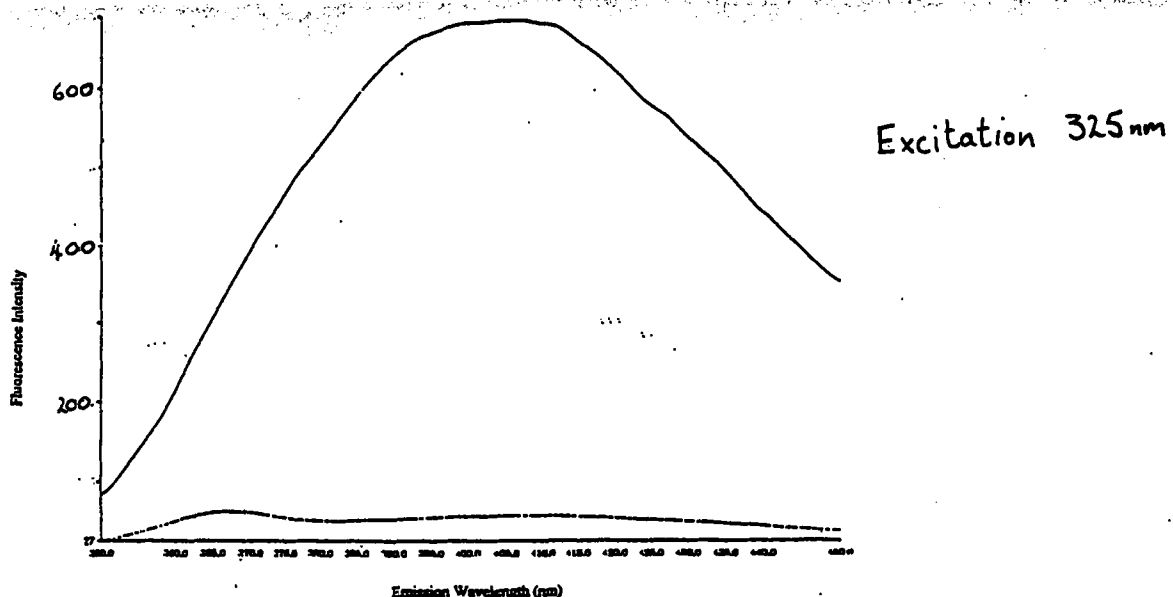


Figure 3.10.1: Emission scans of glycated and non-glycated native HSA at excitation wavelengths of 325nm, 370nm and 360nm.

DISCUSSION

4.1 Glucose Dependency of AGE Production

Many covalent changes take place in proteins on aging [Stadtman, 1988], so it is essential to establish by experiments like those in Section 3.1, that the formation of fluorophores and the fragmentation of proteins are due to reactions of glucose, rather than some other change occurring on the protein during incubations. Throughout the work in this project it has been shown that the formation of fluorescence and the fragmentation of proteins does not occur unless a reducing sugar is present in the incubation medium. Figures 3.1.1, 3.1.2 and 3.1.3 show that fluorophores are not formed in any of the types of HSA used, unless glucose is included in the incubation. Figure 3.1.4 shows that neither native nor delipidated HSA fragment in the absence of glucose. The dependency on glucose of protein fragmentation is not exclusive to HSA, and has been seen in transferrin (Figure 3.4.6) and DSA (Figure 3.2.4).

Le Guen et al (1992) noted considerable increases in the fluorescence of both delipidated and native BSA in the absence of glucose, although when glucose was included in the incubation medium, the increase in fluorescence for each form of BSA was much greater. One possible explanation for the differences between her results and mine could be that Le Guen et al measured fluorescence at excitation 360nm and emission 454nm whilst I used the 'pentosidine wavelength' of excitation 325nm and emission 375nm, which has been shown to be more specific for glucose induced fluorophores than either the wavelengths used by Le Guen et al or the commonly used wavelengths of excitation 370nm and emission 440nm (Section 3.10).

Using BSA as their model protein, Hunt et al (1988b) also showed that the fragmentation of protein was glucose dependent. However, in the absence of glucose, BSA and other proteins have been shown to fragment when exposed to a source of free radicals, such as hydrogen peroxide and lipid hydroperoxides [Hunt et al, 1988b] or to hydroxyl and superoxide radicals derived from a ^{60}Co source [Davies et al, 1987a]. I observed a very small amount of fragmentation in glucose free incubations of transferrin that had high concentrations of copper, but it is possible that Amadori product that had been formed on the protein prior to isolation was involved in this reaction.

4.2 Effect of protein-bound lipid on the production of Amadori product and AGEs in HSA

4.2.1 Effect of bound lipid on Amadori product formation

The production of Amadori product on HSA is not affected by lipid bound to the protein (Figure 3.2.1). Similar experiments by Mereish et al (1982) showed the amount of radioactive glucose bound to HSA to be significantly higher for delipidated HSA than for native HSA. This difference in results can be explained by the experimental conditions used by Mereish (Section 1.12.1).

4.2.2 Effect of bound fatty acid on fluorescent AGE formation

Glucose-induced fluorescence in native HSA is significantly higher than in delipidated HSA, despite the similarity in the amount of Amadori product formed by each of these proteins. This strongly suggests that bound lipid enhances the formation of glycofluorophores. When the experiment was repeated using HEPES buffer instead of phosphate, native HSA no longer had enhanced fluorescence (Section 3.5.6). As HEPES buffer is known to have radical scavenging properties [Halliwell and Gutteridge, 1989], these results suggest that the lipid-dependent fluorophore formation is a free radical mediated reaction.

The finding that delipidated HSA does not produce fluorophores to the same extent as native HSA agrees with work on BSA [Le Guen et al, 1992]. Le Guen et al also suggested that glucose-induced fluorescence on BSA was made up of several components. I have shown that there are at least two components of glycofluorophore production on HSA that has bound lipid; a lipid-independent component and a lipid-dependent component. The change in conformation that HSA undergoes when fatty acids are bound to it [Shaklai et al, 1984] was initially thought to be responsible for the enhanced formation of fluorophores on

native and recombinant HSA. However, it has been shown that the lipid-dependent component of fluorophore formation is a radical mediated reaction, which suggests that glycofluorophore production may also be enhanced by products of lipid peroxidation reactions.

When DTPA was included in the reaction mixture to chelate traces of transition metal found in phosphate buffer, only the fluorophore formation on delipidated HSA was entirely inhibited (Figure 3.3.2). It appears that lipid-dependent fluorophore formation was either still catalysed by the chelated metal ions [Miller et al, 1990], or did not require the presence of transition metal ions. This adds further weight to the conclusion that glucose-derived fluorescence on HSA with bound lipid is made up of more than one component.

Recombinant HSA also forms significantly more glucose-derived fluorophores than delipidated HSA, although it only forms approximately 80% of the fluorescence of native HSA (Figure 3.2.2). Recombinant HSA has a much larger percentage of saturated fatty acids bound to it than native HSA [Delta Biotechnology Limited (Data Sheet); Saifer and Goldman, 1961]. The inability of saturated lipids to undergo peroxidation reactions may be responsible for the lower amounts of fluorophore produced by the recombinant HSA [Halliwell and Gutteridge, 1989]. An additional explanation for the enhanced production of fluorophores by native HSA is that the protein has become glycated *in vivo* prior to isolation from serum which may enhance the initial rate of fluorophore production. The lower levels of copper bound to recombinant HSA could be a further possible reason for the depressed fluorophore formation (See Section 4.4.1).

4.2.3 Effect of bound fatty acid on protein fragmentation

Glucose-induced fragmentation of HSA does not appear to be affected by bound fatty acids (Figure 3.2.3). This was unexpected as I found that lipid enhances glycofluorophore formation via a radical mediated reaction. It is also known that free radicals [Davies et al,

1987a] and lipid peroxides [Hunt and Dean, 1989] can cause protein fragmentation, and it has been shown that the glucose-induced fragmentation of HSA is a free radical mediated reaction (Section 3.5.2). Figure 3.2.3 shows that any free radicals formed by interaction with the lipids are not in a position to cleave the protein backbone. These radicals may react either with the lipid itself, or at a site on the protein which is not vulnerable to fragmentation, possibly resulting in fluorophore formation.

When the fragmentation of both native and delipidated DSA was investigated it was found that unlike HSA, the two forms of dog albumin did not fragment identically (Figure 3.2.4). Contrary to expectations, it was the delipidated DSA which had enhanced fragmentation rather than the native DSA. Clearly, bound lipid does not play a catalytic role in fragmentation of HSA, and in addition it somehow protects DSA from glucose-induced fragmentation. Two possible reasons why delipidated DSA is more vulnerable to glucose-induced fragmentation are:

- (i) the removal of fatty acids exposes amino acid residues which can be oxidised to produce free radicals that can lead to cleavage of the protein backbone [Wolff and Dean, 1986] and
- (ii) delipidation of albumin is known to cause a conformational change in the protein (where the C-terminal becomes less closely packed) [Dröge et al, 1988]; this could make parts of the protein backbone more vulnerable to either radical attack or attack by proteases.

The possible mechanisms of cleavage of the protein backbone will be discussed further in Section 4.10.2.

4.2.4 Aggregation

Only one further contrast between native and delipidated serum albumins showed up in this project. This can be seen in Figures 3.4.12 and 3.4.13 where the two proteins have been incubated in a phosphate-buffered glycation system with additional Fe^{III} ions. Where the

concentrations of iron are high, the native HSA appears to form some high molecular weight products and the delipidated HSA does not. Malondialdehyde (MDA) is a secondary product of lipid peroxidation which is a very potent protein cross-linker [Halliwell and Gutteridge, 1989]. It is possible that where concentrations of iron are very high, the lipids bound to native HSA, which may already have undergone initial reactions of lipid peroxidation [Fletcher and Tappel, 1971], react further to form MDA. As only 6% of the lipids bound to native albumin have the capacity to readily undergo lipid peroxidation reactions [Saifer and Goldman, 1961], the effect observed here is probably a minor reaction observed only under extreme reaction conditions such as at very high concentrations of iron ions.

4.3 Effect of trace transition metals on AGE formation

Both fluorophore formation and fragmentation of proteins were shown to require the presence of catalytic amounts of transition metals. Phosphate buffer contains traces of copper and iron which were thought to be catalysing the formation of AGEs. To demonstrate this, DTPA was added to the incubations to try to prevent the copper and iron from acting as catalysts of oxidation reactions.

4.3.1 Fluorescence

Fluorescence inhibition was 100% only for the delipidated HSA. Fluorescence of native HSA was inhibited by only 88% and recombinant HSA by only 63%. Since the DTPA (1mM) is well in excess of the trace contaminants of Cu^{II} (4 μM) and Fe^{III} (5 μM) it is unlikely that there were any unchelated metal ions in solution. Fu et al (1992) also found incomplete inhibition of fluorescence development at the same wavelengths, in collagen incubated with glucose and DTPA. This "leakage" could be because metal ions chelated to DTPA still retain a certain amount of their catalytic ability. Transition metals, and in particular iron, have been shown to retain some of their ability to catalyse oxidation reactions whilst bound to DTPA. Neither the chelators DTPA or desferrioxamine are able to bind metals in completely inert complexes and there does not appear to be any chelator capable of entirely blocking the catalytic abilities of these metals [Miller et al, 1990]. It is interesting to note that whilst the formation of fluorophores on delipidated HSA is entirely inhibited by the addition of DTPA, both the native and recombinant HSAs (which both have bound lipid) still form some fluorescence when DTPA is included in the incubation medium. One possible explanation for this is that the chelated transition metals are unable to catalyse the formation of lipid-independent fluorescence, but that they retain enough catalytic activity to catalyse the formation of lipid-dependent fluorescence.

4.3.2 Fragmentation

Fragmentation was completely inhibited by the addition of DTPA for all of the proteins investigated (Figures 3.3.4 and 3.4.6), showing that the process involves a transition-metal-catalysed reaction which may be a radical generating reaction such as the Fenton reaction. This finding is in agreement with Hunt et al (1988b) where complete inhibition of the sugar-induced fragmentation of BSA in a phosphate-buffered incubation was observed when DTPA was added to incubations.

4.4 Effect of Cu^{II} and Fe^{III} on AGE formation

Both Cu^{II} and Fe^{III} ions are present as trace contaminants in sodium phosphate buffer, so in order to see whether the catalytic effect seen was due to one or other of these metals, additional quantities of Cu^{II} and Fe^{III} ions were added to the incubations.

4.4.1 Effect of Cu^{II} on fluorescent AGE formation

When additional Cu^{II} ions are added to the phosphate-buffered glycation system used in Figure 3.2.2, only the recombinant HSA has enhanced fluorophore formation (Figure 3.4.1). No enhancement of fluorophore production in either of the HSAs purified from human serum is observed. Recombinant HSA is produced in a very low copper environment so it does not have as much bound copper as the other forms of HSA derived from serum [Delta data sheet]. *In vivo* there is less than 1 mole of copper bound per mole of HSA [Naik et al, 1975], but it is possible that more copper becomes bound to HSA during its purification process. Copper bound to HSA has been shown to retain its capacity to catalyse oxidation reactions [Halliwell and Gutteridge, 1989]. It is possible that at high glucose concentrations (500mM), both the native and the delipidated HSA have sufficient copper (both from the contaminants in the phosphate buffer and the copper that is already bound to the protein) for the reaction to proceed at the maximum rate for that particular set of reaction conditions.

When additional Cu^{II} is added to the incubations, recombinant HSA produces as much fluorophore as the native HSA. This implies that a deficiency in bound copper in recombinant HSA is the reason for the lower production of fluorophores seen in Figure 3.2.2 rather than any difference in the composition of the bound lipid or the protein itself (see Section 4.2.2).

In an attempt to see the effect of Cu^{II} ions more clearly, native and delipidated HSA and transferrin were incubated with a much lower concentration of glucose (25mM rather than 500mM) with additional CuSO_4 added to the media. The addition of Cu^{II} ions had no effect on the production of fluorophores on transferrin except at very high concentrations of Cu^{II} . However, both delipidated and native HSA showed dose-dependent increases in fluorophore production with additional Cu^{II} . This indicates that at low concentrations of glucose the Cu^{II} catalysed step becomes rate limiting. HSA binds copper very strongly at a specific binding site (see Section 1.11.4), and although transferrin has the ability to bind copper it does not do so as strongly as HSA. This could be a factor influencing the amount of glycofluorophore formed by each. This will be discussed at greater length in Section 10.1. HEPES buffer has much lower concentrations of transition metal contaminants than phosphate buffer. Work done using HEPES buffer (see Section 3.5) shows that even at high glucose concentrations, fluorophore formation is boosted by the addition of further copper in all three forms of HSA.

4.4.2 Effect of Cu^{II} on sugar-induced protein fragmentation

When additional copper was added to the glycation media of HSA, fragmentation occurred. The fragments produced can be seen as broad diffuse bands in Figure 3.4.5. The approximate masses of these bands are 15 and 26 - 33kD. The 26 - 33kD band shows more intense staining at 26 and 33kD than in the intermediate area. Pepsin digests of BSA produces fragments of 16, 29 and 34kD [King, 1973]. The 34kD fragment found by King corresponded to the N-terminal portion of the molecule and the 29kD fragment to the C-terminal. Hunt et al (1988a, 1993) obtained three discrete bands with approximate masses of 29, 37 and 48 kD, and found no evidence of the non-specific protein fragmentation (such as the smearing that is seen in lanes D and H of Figure 3.4.5). Previous attempts by Ahmed (1992) to reproduce the fragmentation pattern of Hunt et al produced no discrete bands. Incubations of both BSA and HSA with Cu^{II} and ascorbate produced fragments of 3, 18, 22, 47 and 50kD [Marx and Chevion, 1985], and it was postulated that these specific

cleavage sites were linked to low-affinity Cu^{II} binding sites. The masses of the fragments obtained in Figure 3.4.5 correspond most closely to those found by King using pepsin digestion. Although the fragmentation observed is glucose-dependent, there may be traces of proteases in preparations of HSA [Peters, 1985] which could cause protein fragmentation. It is possible that much of the cleavage observed here occurs in the region between sub-domains IIA and IIB, which would result in the formation of two fragments of similar size, such as the fragments of 26 and 33kD which I found. For more precise identification of the cleavage sites it would be necessary to sequence parts of each fragment and then to compare them with the three dimensional structure of HSA.

Transferrin which has been incubated with glucose and additional Cu^{II} ions in a phosphate-buffered system shows very specific fragmentation. Four bands with approximate masses 16, 32, 41 and 62kD are produced (lane D in Figure 3.4.6). Jhoti et al (1988) isolated an 18kD fragment by subtilisin digestion of ovotransferrin. This retained the capacity to bind iron and corresponded to domain II of the N-terminal lobe. Tryptic digests of human transferrin produced fragments of masses 43, 36, and 33.5 kD [Evans and Williams, 1978]. Both the fragments of 43 and 36 kD retained their iron binding capacities and it was found that the 36 kD fragment was from the N-terminal and the 43kD fragment was from the C-terminal. The two lobes of transferrin are very similar except the C-terminal has both of the carbohydrate moieties, each of mass 2.2kD, attached to it. This may account for the difference in mass between the two fragments. MacGillivray et al (1982) obtained a 35kD fragment of human transferrin by thermolytic digestion. This fragment retained its iron binding capacity. It is possible that the fragments that I obtained are products of cleavage at two specific sites. The first of these sites is likely to be on the peptide joining the two lobes of transferrin and the second cleavage site could be between the two sub-domains on the N-terminal lobe. The fragments of 41 and 32 kD are probably the result of cleavage of the short peptide linking the two lobes of the transferrin molecule, and the fragments of 15 and 62kD the result of cleavage between the two sub-domains on the N-terminal. It is unlikely that the fragment of 15kD could be due to the cleavage between the C-terminal sub-domains as the C-terminal domain has both carbohydrate moieties attached to it and

would therefore give fragments of greater mass. As with HSA, it would be necessary to carry out amino acid analysis in order to accurately identify the cleavage sites on transferrin.

It can be seen in Figures 3.4.7 and 3.4.8 that there is a dose-dependent increase in glucose-induced fragmentation of native HSA and transferrin with added copper. The fragmentation in these gels is only slight, but the amount of smearing produced by native HSA and the intensity of the 32kD fragment of transferrin both increase with copper concentration.

4.4.3 Effect of Fe^{III} on fluorescent AGE formation

Iron^{III} has the capacity to catalyse spin-forbidden oxidation reactions and is the most common catalyst for biological free radical mediated processes [Miller et al, 1990; Halliwell and Gutteridge, 1989]. It is also one of the principal transition metal contaminants found in phosphate buffers along with Cu^{II} ions. Unlike copper, iron does not bind strongly to HSA, but binds strongly to two sites on human transferrin. Work was therefore carried out with Fe^{III} to elucidate what role is played by the tight binding of transition metals to proteins in the formation of glycoxidative products.

The formation of glycofluorophores on native and delipidated HSA increased on adding FeCl₃ at concentrations of 10μM (see Figures 3.4.9 and 3.4.10). Beyond this concentration, further amounts of FeCl₃ had little effect on the formation of fluorophores except at 200μM where a small increase was observed in the amount of fluorophore produced by delipidated HSA. This effect is very different to the dose-dependent increase in fluorophore formation seen in both delipidated and native HSA when Cu^{II} is the species of transition metal ion added to the incubation medium.

Transferrin exhibited a clear dose-dependent increase in fluorophore formation with increasing concentrations of FeCl₃ (Figure 3.4.11). A higher range of concentrations of FeCl₃ was used in incubations of transferrin to see if fragmentation could be induced by high concentrations of Fe^{III} ions. Even at the lower concentrations of FeCl₃, which overlapped with those used in the incubations of delipidated and native HSA, i.e. 100μM and 200μM of

FeCl_3 , increases in the amount of fluorophore formed were seen. An equivalent increase in fluorophore formation was not observed for the samples of HSA at these concentrations of FeCl_3 . When it is taken into account that there is no dose-dependent increase in transferrin fluorophore formation with Cu^{II} , it can be concluded that the specific binding of metals to proteins may in some way enhance fluorophore formation.

4.4.4 Effect of Fe^{III} on glucose-induced fragmentation

Neither delipidated or native HSA fragment when Fe^{III} ions are included in the incubation media (Figures 3.4.12 and 3.4.13), unlike the fragmentation of both forms of HSA that is promoted by additional Cu^{II} in the glycation incubations (Figure 3.4.5, Lanes D and H). More surprising is the lack of fragmentation of transferrin when Fe^{III} is added to the incubation rather than Cu^{II} (Figure 3.4.6, Lanes F and D respectively). This contrasts with the increase in fluorescence formation seen when samples of transferrin are incubated with FeCl_3 .

4.4.5 Summary

Iron^{III} ions are able to catalyse the formation of glycofluorophores on transferrin, but not on HSA and Cu^{II} ions catalyse glycofluorophore development in HSA, but not in transferrin. It therefore appears that the tight binding of metal ions to proteins can catalyse the formation of glycofluorophores. When the fluorescence data is combined with the fragmentation data it can be seen that the specific binding of transition metal to transferrin does not play a role in the catalysis of protein fragmentation and that of the two transition metals examined, only copper is capable of catalysing this reaction. Contrary to the idea put forward by Ahmed (1992), the high-affinity binding of transition metals to proteins does not appear to play any role in the catalysis of protein fragmentation.

4.5 Effect of choice of buffer on AGE formation

It was initially thought that phosphate-buffered glycation systems favour glycoxidative reactions because they contain traces of the transition metals copper and iron [Stadtman, 1990]. This conclusion was confirmed by the inhibition of fragmentation, and the partial inhibition of glycofluorophore production by DTPA (Section 4.3). An alternative buffer, HEPES, contains very low amounts of transition metal contaminants compared to phosphate buffer, and it is also thought to have radical scavenging properties [Halliwell and Gutteridge, 1989].

When the three forms of HSA are incubated in a HEPES-buffered glycation system (Figure 3.5.4) under otherwise identical conditions to those used in the phosphate buffered glycation system (Figure 3.2.2), a different pattern of fluorescence production emerges. In the phosphate system both native and recombinant HSA produce significantly more fluorescence than delipidated HSA. The fluorescence of delipidated HSA appears to remain essentially unchanged when the buffering system is changed from phosphate to HEPES. However, in HEPES both recombinant and native HSA produce much less fluorescence than in phosphate. In fact they both produce similar amounts of fluorescence to delipidated HSA, showing that it is only the lipid-dependent component of their fluorescence that is inhibited by the HEPES buffer. As HEPES buffer can act as a radical scavenger it seems likely that the formation of lipid-dependent glycofluorophores is a free radical mediated reaction.

Transferrin was selected for comparing fragmentation in different buffers (Figure 3.5.5) because of the clear fragmentation pattern it produces in phosphate buffered glycation systems where additional Cu^{II} is included (Figure 3.4.6). However, in the HEPES buffered glycation system there was much less fragmentation and a different distribution of fragments, even though enough copper was added to increase the concentration of metal ions above the level found in the phosphate-buffered incubations. The limited fragmentation

of transferrin seen in Figure 3.5.5 shows that the free radical mediated cleavage of transferrin at certain sites can be inhibited by HEPES. Previous work done on fragmentation [Davies, 1987; Hunt et al, 1988a], shows that this is a radical mediated reaction, which is in agreement with results obtained here. Other buffers with radical scavenging properties include Tris, Mes and Mops [Halliwell and Gutteridge, 1989] and TAPSO [Fu et al, 1992]. Therefore, choice of buffer should be an important consideration when designing experiments investigating glycoxidative reactions.

4.6 Role of Glucose Autoxidation

Wolff and Dean (1987) have postulated that up to 45% of the products detected by common glycation assays is formed not through the Amadori rearrangement of a Schiff's-Base (Figure 1.1), but through the reaction of an α -ketoaldehyde, formed by autoxidation of glucose (Figure 1.3), with an amino group. Unfortunately Wolff was unable to separate the possible contributions to AGE formation of glucose autoxidation products and Amadori products. However, by preincubating glucose in the absence of protein prior to incubation with type 1 corneal collagen, Chase et al (1991) were able to show the effect that glucose autoxidation has on the formation of collagen aggregates. A group of experiments investigating the effect of preincubating glucose in the presence of CuSO_4 was carried out to see whether the autoxidation of glucose played a significant role in the development of fluorescent AGEs on HSA.

The experiments carried out in Section 3.6 looked at the effect of glucose preincubated in the presence of CuSO_4 on fluorescence produced by native, delipidated and recombinant HSA (Figures 3.6.1, 3.6.2 and 3.6.3). Development of fluorescence in native HSA was essentially unchanged by the use of preincubated glucose, but delipidated HSA generated more fluorescence during the early stages of the reaction. The lag phase normally seen with delipidated HSA was absent whether glucose was preincubated with or without additional Cu^{II} . This shows that products formed during glucose preincubation play an important role in glycofluorophore formation on delipidated HSA, at least during the first two days (under the reaction conditions used here). Chase et al (1991) showed that over three days, collagen incubated with preincubated glucose had 3-fold higher aggregation than collagen incubated with fresh glucose. The effect of glucose preincubation on glycofluorophore production in delipidated HSA (Figure 3.6.2) was much less than that of collagen. This suggests that glucose autoxidation products are more important in the formation of collagen aggregates than in the formation of glycofluorophores on delipidated HSA.

The rate of fluorophore production in delipidated HSA after the lag phase of two days is identical, implying that after the initial two days the same mechanism of fluorescence formation is occurring in both types of samples. Therefore it is only in the initial part of this reaction that the effects of glucose oxidation products can be seen clearly.

Unfortunately it is not possible to tell from these experiments whether the mechanism of fluorophore formation after the lag phase is via an Amadori product intermediate or via the glucose autoxidation pathway (Figures 1.1 and 1.3). As it takes several days for the Amadori product to form [Baynes et al, 1984], a lag phase such as the one seen here would be expected even if fluorophore formation was occurring solely via the Amadori product. It is also possible that the lag phase is due to the time it takes for the glucose to autoxidise (either during the preincubation period or during the first two days of incubation with the protein). This lends weight to the idea that glucose autoxidation may play a significant role in the formation of AGEs. However, going against this idea is the observation that Amadori product formation is unaffected by the presence of the metal chelator DTPA [Fu et al, 1992]. Once the Amadori product forms it is unlikely that glucose autoxidation is going to be a major reaction as Amadori product is autoxidised much more readily than glucose [Gillery et al, 1988; Azevedo et al, 1988]. This would mean that if fluorescence was forming via an autoxidative reaction, then it would be more likely to be via the autoxidation of Amadori product than of glucose. Therefore, despite the initial role the products of glucose autoxidation play in the formation of glycofluorophores, it is unlikely that the major route for the production of glycofluorophores is via glucose autoxidation.

4.7 Differences between Glucose and Fructose

Nearly all of the work in this project looked at reactions involving glucose as the reducing sugar. Throughout the literature, differences of reactivity of various reducing sugars have been shown [Bunn and Higgins, 1981]. In Section 3.7 it was shown that native HSA gave low readings in the periodate assay when incubated with either 10mM glucose or 10mM fructose, and high readings when incubated with 500mM glucose. Native HSA incubated with 10mM glucose produced no significant increase in fluorescence, but in 500mM glucose and 10mM fructose (Figures 3.7.2 and 3.7.3), a large increase in fluorescence was seen. The fluorescence produced by incubation with 10mM fructose is much greater than that produced by 500mM glucose.

It seems that in the case of protein modification by fructose, there is little relation between the formation of Amadori compounds and fluorophore formation. Several factors may contribute to this. Firstly, in order to be detected by the periodate assay, sugar-protein adducts must have a carbonyl carbon adjacent to a carbon carrying a hydroxyl group, so that formaldehyde can be liberated and detected colorimetrically [Furth, 1988]. Therefore no cleavage of the carbonyl group will occur in the Heynes product due to the absence of hydroxyl groups on the carbon adjacent to the carbonyl group [Furth, 1988] and hence the Heynes product is going undetected. McPherson et al (1988) found that approximately 15% of the "Amadori product" formed from fructose had in fact the same structure as the glucose Amadori product. This suggests that glucose has been formed through the enolisation of fructose, and is reacting with the protein.

Alternatively, 3-deoxyglucosone can be formed directly from fructose without passing through an Amadori-type intermediate [Shin et al, 1988]. This therefore opens up a new (and rapid) route through which fructose can produce large amounts of glycofluorophores whilst bypassing the formation of Heynes product. Igaki et al (1990) showed that 3-deoxyglucosone was a likely intermediate in the formation of fluorophores, and recently

Requena et al (1993) suggested from inhibition studies using aminoguanidine that a carbonyl intermediate such as 3-deoxyglucosone was involved in the formation of glycofluorophores. It has been shown that 3-deoxyglucosone can be formed from the Amadori product of glucose. Therefore, in incubations with glucose, a build-up of Amadori product would be needed before glycofluorophores could be formed via 3-deoxyglucosone.

It is difficult to say which of these possible explanations for the disparity between glycofluorophore production and observed Amadori product formation is the most important. Evidence has recently accumulated for the role played by carbonyl intermediates in the production of glycofluorophores [Igaki et al, 1990; Kato et al, 1989; Requena et al, 1993]. Coupled with the ability of 3-deoxyglucosone to be formed directly from fructose [Shin et al, 1988], it is likely that much of the glycofluorophore formation from fructose does not involve the Heynes product as an intermediate.

4.8 Effect of inhibitors on glycofluorophore formation

To elucidate reaction mechanisms, the potential of four compounds to act as inhibitors of glycofluorophore production was examined. Two of the compounds tested were the free radical scavengers propyl gallate and trolox (a water-soluble derivative of vitamin E). Free radical scavengers were chosen as potential inhibitors of fluorophore formation due to the implications that some of the reactions are free radical mediated processes (see Section 4.5). The other two compounds, aminoguanidine and acetyl salicylic acid, have previously been studied as potential inhibitors of glycoxidative reactions [Requena et al, 1993; Swamy and Abraham, 1989].

4.8.1 Aminoguanidine

Aminoguanidine is the most effective inhibitor of fluorophore formation of the four compounds tested. It is a hydrazine compound with three free amino groups (Figure 1.5). Various mechanisms have been postulated for the way in which aminoguanidine acts as an inhibitor, but all the more recent evidence has pointed to a mechanism in which aminoguanidine reacts with a dicarbonyl intermediate of the Maillard reaction [Requena et al, 1993; Edelstein and Brownlee, 1992], and thus prevents fluorophore formation and protein crosslinking.

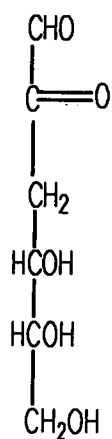
Aminoguanidine gave a dose-dependent inhibition of fluorophore formation when included in incubations of all three proteins used in this study (Figure 3.8.1). Unlike the other three compounds tested, aminoguanidine did not produce any background fluorescence of its own when incubated with protein in the absence of glucose, even when the maximum concentrations of aminoguanidine was used. Very low concentrations of aminoguanidine (25 μ M) reduced the fluorophore formation in both forms of HSA by over half. However, in transferrin the inhibition of fluorophore formation did not begin until the concentration of

aminoguanidine reached 250 μ M. If the mechanism of inhibition involved direct reaction between aminoguanidine and glucose, as suggested by Lewis and Harding (1990), then the inhibition of transferrin fluorophore formation would resemble that of HSA. As the concentration of glucose was identical in all three sets of incubations it can be seen that aminoguanidine inhibition of glycofluorophore production does not involve direct interaction with glucose.

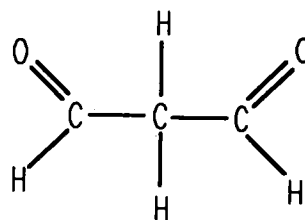
There is evidence to suggest that the lipid-dependent fluorophores are formed via a radical mediated process (see Section 4.5), but as aminoguanidine is not known to be a radical scavenger it is probably inhibiting another step in the formation of lipid-dependent fluorophores. The formation of lipid-dependent glycofluorophores on the native HSA was also inhibited, suggesting that there may be interaction of protein or bound lipid with a secondary product of lipid peroxidation, such as malondialdehyde (MDA) [Halliwell and Gutteridge, 1989]. Aminoguanidine would then be able to inhibit this reaction in a similar way to reactions mediated by 3-deoxyglucosone. MDA, like 3-deoxyglucosone, is a dicarbonyl compound which has been shown to produce fluorophores when incubated with proteins [Kikugawa and Beppu, 1987]. Aminoguanidine is a potent scavenger of 3-deoxyglucosone [Oimomi and Igaki, 1989; Edelstein and Brownlee, 1992] so due to the structural similarities between 3-deoxyglucosone and MDA (Figure 4.1) it is conceivable that the lipid-derived fluorophore formation is blocked by the scavenging of MDA.

4.8.2 Propyl gallate

Propyl gallate is an effective synthetic free radical scavenger with the capacity to bind transition metals [Halliwell and Gutteridge, 1989]. When each of the proteins is incubated with high concentrations of propyl gallate in the absence of glucose, there is an increase in fluorescence production. However, this increase is smaller than the increase in fluorescence seen when each of the proteins is incubated with only glucose.



3-Deoxyglucosone



MDA

Figure 4.1: Structures of 3-deoxyglucosone and MDA

Inhibition of glycofluorophore production is seen in all three proteins (Figure 3.8.2) when the concentration of propyl gallate is at its lowest (25 μ M). It is the native HSA which has the largest amount of inhibition of glycofluorophore production at this low concentration of propyl gallate. Only a very small increase in the amount of inhibition is seen in native HSA beyond a propyl gallate concentration of 25 μ M, and beyond 250 μ M no further inhibition is observed. Delipidated HSA and transferrin also show inhibition of fluorophore production at 25 μ M propyl gallate, but this is not nearly as large as for native HSA. Delipidated HSA and transferrin show a large decrease in the amount of fluorophore produced when the concentration of propyl gallate is increased to 250 μ M. Clearly, the additional lipid-derived fluorescence of native HSA is inhibited at very low concentrations of propyl gallate, showing that this radical mediated process is readily inhibited by the propyl gallate. However, as the concentration of propyl gallate rises, the amount of background fluorescence also rises to the amount obtained for each of the proteins incubated with propyl gallate in the absence of glucose.

Fluorophore formation that is not lipid-dependent has been shown not to be affected by the radical scavenging properties of HEPES (Section 4.5). The mechanism for inhibition of this type of fluorophore formation by propyl gallate is less clear. It is possible that propyl gallate is a more effective radical scavenger than HEPES and is therefore able to scavenge the radicals involved in non-lipid-dependent fluorophore formation, but it is also possible that the metal chelating properties of propyl gallate are sufficient to prevent the metal-catalysed oxidation reactions required for fluorophore formation from occurring.

4.8.3 Trolox

Vitamin E (α -tocopherol) has been tested as a potential inhibitor of protein glycation [Ceriello et al, 1988], and it has also been used as an inhibitor of lipid peroxidation that was initiated by a glycated peptide-Fe complex [Sakurai et al, 1991]. Trolox, a water soluble derivative of α -tocopherol, was used so that the use of ethanol as a solvent could be avoided, and the reaction conditions for all four potential inhibitors would be identical.

Trolox is a free radical scavenger (Figure 1.5) although unlike propyl gallate it is not known to be a metal chelator [Halliwell and Gutteridge, 1989].

Trolox incubated with each of the proteins in the absence of glucose generates a large amount of fluorescence. However, at low concentrations of trolox, inhibition of fluorophore formation is visible in both native and delipidated HSA (Figure 3.8.3). As the concentration of trolox rises the amount of background fluorescence also rises, thereby masking the inhibition seen. However, unlike delipidated HSA, the amount of fluorescence produced by native HSA with 25mM trolox in the presence of glucose falls well short of the amount of fluorescence produced in the absence of trolox. This shows that inhibition of the lipid component of glycofluorophore production can be seen despite the large levels of background fluorescence produced by the trolox. It is likely that this reaction is being inhibited by the radical scavenging abilities of trolox. The inhibition of lipid-independent fluorophore production seen in both forms of HSA is unlikely to be due to the radical scavenging properties of trolox, as fluorophore formation on delipidated HSA has been shown to be unaffected by incubation in HEPES (Section 4.5) and no inhibition by trolox of glycofluorophore formation on transferrin is seen. Therefore, the inhibition of this component of the fluorescence could be due to the prevention of Amadori product formation on HSA [Ceriello et al, 1988], which in turn would lead to the prevention of the formation of the fluorophore precursor 3-deoxyglucosone.

4.8.4 Acetyl Salicyclic Acid

It had been hoped that the blocking of Amadori product formation would lead to a reduction in glycofluorophore production and hence give evidence that glycofluorophore production on proteins proceeds via an Amadori intermediate. Unfortunately, unless a method of separating the fluorescence produced by acetyl salicyclic acid from that produced by glucose is developed, the very high amounts of fluorophore produced by acetyl salicyclic acid prevent it from being a useful inhibitor in mechanistic studies such as these.

4.8.5 Summary

Of the four compounds investigated here for their potentials as inhibitors of glycofluorophore formation, aminoguanidine was the most effective and did not produce background fluorescence. Inhibition of glycofluorophore production in all three proteins was also seen when propyl gallate was included in the incubations. Trolox inhibited fluorophore formation in native and delipidated HSA, but did not inhibit fluorophore formation in transferrin. The extremely high background fluorescence produced by acetyl salicylic acid completely masked the fluorescence produced by glucose and proteins. The information that was obtained from these experiments is discussed with respect to the possible mechanisms of fluorophore formation in Section 4.10.1.

4.9 Effect of Varying Concentrations of protein and glucose

Many different concentrations of both glucose and proteins have been used throughout the literature on glycoxidative reactions. As reaction rates are dependent on both absolute and the relative concentrations of reactants, I decided to investigate the effect of these variables on glycofluorophore formation. Transferrin was chosen as the model protein as it has no lipid-dependent component of fluorescence, making the production of glycofluorophores a simpler reaction to study than in native HSA (See Section 4.2). Also, unlike HSA, which gets glycated at several sites, transferrin does not get as heavily glycated [Ney et al, 1985], so there will be less variation in the glycated proteins that undergo reactions of fluorophore formation.

If it is assumed that Amadori product is a precursor of glycofluorophores, then a possible rate determining step in glycofluorophore production is the formation of Amadori product. Unless glucose is in the straight chain form, there is no reactive carbonyl group on it, therefore the amount of sugar in the acyclic form may affect the rate at which the Schiff's base is formed. Hence the production of Amadori product is dependent on the amount of glucose present in the acyclic form, providing that the Amadori rearrangement is not the rate determining step.

The amount of fluorophore formed by transferrin (incubated at concentrations of 40, 20, 10 and 1mg/ml) with glucose at a concentration of either 25 or 100mM is greatest when the concentration of transferrin in the incubation is at its lowest (i.e. 1mg/ml). In Figure 3.9.1 it can be seen that the amount of fluorophore produced decreases with increasing concentrations of transferrin in the incubations, implying that it was the transferrin that was in excess over the glucose. Despite the seemingly large excess of glucose over protein (Tables 3.9.1 and 3.9.2), if the percentage of glucose in the reactive acyclic form is taken to be 0.002% [Bunn and Higgins, 1981], it can be seen that the protein does indeed exist in large molar excess over the concentration of reactive species of glucose.

However the production of glycofluorophores is not simply dependent on the relative concentrations of protein and glucose. The same relative concentration of reactants exists when the protein concentration is 40mg/ml and the glucose concentration is 100mM, as when the protein is incubated at 10mg/ml with 25mM glucose. However, the amount of fluorescence produced by the sample of protein incubated at 40mg/ml is ten times higher than that produced by the incubation at 10mg/ml. This shows that the rate of fluorophore production increases with the absolute concentrations of the reactants, as would be expected.

From this work it can be seen that both the relative and absolute concentrations of reactants (protein and sugar) can affect the formation of glycofluorophores. Therefore it is not possible to make comparisons between sets of data that have been obtained under non-identical experimental conditions. This should be especially borne in mind when extrapolating *in vitro* models to an *in vivo* situation.

4.10 Mechanisms of AGE formation

From the results obtained in this thesis certain conclusions about the mechanisms involved in the formation of glycofluorophores and the sugar-induced fragmentation of proteins can be reached.

4.10.1 Mechanisms of glycofluorophore formation

i) Role of metal-catalysed oxidation

Fluorophore production on HSA has at least two components, a lipid-dependent and a lipid-independent component, both of which are formed in a transition metal catalysed reaction. Theoretically, all metal-catalysed oxidation involves free radicals [Morrison and Boyd, 1987]. However, the lipid-independent component of glycofluorophore production, whilst metal catalysed, appears to be largely unaffected by the free radical scavenger HEPES, although some inhibition is seen in delipidated HSA incubated with trolox or propyl gallate. This may either be due to these compounds being more effective radical scavengers than HEPES or to trolox blocking the formation of Amadori product (Section 4.8.3) and propyl gallate chelating the transition metals. The formation of fluorophores is a metal catalysed reaction, which is enhanced by the high affinity binding of transition metals to proteins. It is likely that if the free radicals produced by metal-catalysed oxidation act at their site of formation, they may not be completely scavenged by either the HEPES buffer or the trolox.

The lipid-dependent component of glycofluorophore production is completely inhibited by HEPES, trolox and propyl gallate showing that this reaction is indeed a free radical mediated process. This is in agreement with those who observe inhibition of glycoxidative reactions when radical scavengers are included in incubations [Le Guen et al, 1992; Fu et al, 1992; Hunt and Wolff, 1991].

The fluorescent modification of HSA by peroxidation of bound lipids was reported by Fletcher and Tappel (1971) although this was observed in the absence of glucose. It seems likely that the lipids bound to commercially available HSA have already undergone some peroxidation reactions, and it is perhaps the reaction of these products with glucose that we see in a phosphate-buffered glycation system.

ii) Role of glucose autoxidation products

The glucose autoxidative pathway was put forward by Wolff and Dean (1987) as having a major role in the formation of AGEs. In Section 3.6, it was shown that the formation of glycofluorophores on delipidated HSA was enhanced by products of glucose oxidation reactions. Unfortunately it is not possible to determine the contribution of this reaction beyond two days as the rates of fluorophore production are identical in all samples after this time. It is unlikely that glucose autoxidation is the main route by which glycofluorophores are formed, as fluorophore production can occur in HEPES buffer. Further evidence that fluorophore formation may not occur via the glucose autoxidation pathway is that the Amadori product is formed through a non-metal catalysed reaction pathway [Fu et al, 1992], that Amadori products autoxidise preferentially to glucose [Gillery et al, 1988; Azevedo et al, 1988], and that fluorophores form via a radical mediated reaction from glycated proteins in the absence of glucose [Le Guen et al, 1992].

iii) Role of dicarbonyl intermediates

The experiments carried out in Sections 3.8 and 3.7 provide indirect evidence for a dicarbonyl intermediate in the formation of glycofluorophores. Aminoguanidine is not a free radical scavenger, but is postulated to scavenge dicarbonyl compounds such as 3-deoxyglucosone [Requena et al, 1993; Edelstein and Brownlee, 1992; Oimomi and Igaki, 1989]. Furthermore, in Section 3.7 it was shown that fructose is a very potent producer of glycofluorophores (when compared to glucose). This can be explained by the ability of fructose to form 3-deoxyglucosone directly, without going through the Heynes product [Shin et al, 1988].

There is now increasing evidence that 3-deoxyglucosone may play a role in the crosslinking of proteins *in vivo*. Until recently, the crosslinking properties of 3-deoxyglucosone had only been shown to exist *in vitro* [Kato et al, 1987], but a recent publication shows that it can be detected in the serum and levels of it are elevated in diabetics and especially so in those with nephropathy [Niwa et al, 1993].

4.10.2 Mechanisms of sugar-induced protein fragmentation

Protein fragmentation has been shown here to be metal catalysed (Section 4.3) because addition of the transition metal chelator DTPA completely inhibited the glucose induced fragmentation. Similarly, the fragmentation of apoprotein B when LDL was incubated under oxidative conditions was inhibited by the metal chelator EDTA [Fong et al, 1987], and both EDTA and DTPA were found to inhibit the fragmentation of proteins incubated with model Amadori compounds [Kawakishi et al, 1990], or with hydrogen peroxide [Wolff and Dean, 1986].

Previous work has shown fragmentation of HSA in a glycation medium which included Cu^{II} [Hunt et al, 1988a]. Hunt also claimed to have observed sugar-induced fragmentation by Fe^{III} , although the results were not actually shown. My own experiments carried out under identical conditions, using Fe^{III} as the catalytic transition metal, showed no fragmentation of HSA. The iron binding protein transferrin was investigated to see whether fragmentation is caused by the high-affinity binding of transition metals to specific sites, since HSA (a copper binding protein) fragmented in the presence of Cu^{II} , but not Fe^{III} . However, transferrin did not fragment in the presence of iron either, showing that tight binding of transition metal to protein is not necessary for protein fragmentation. Of the two transition metals investigated, only Cu^{II} appears to catalyse protein fragmentation.

Work done using HEPES buffer (Section 4.5) shows that glucose-induced fragmentation is a radical mediated reaction. Similarly, the oxidative fragmentation of LDL was inhibited by the radical scavenger vitamin E [Fong et al, 1987] and the glucose induced fragmentation of BSA was inhibited by the hydroxyl radical scavengers benzoic acid, deoxyribose and sorbitol [Hunt et al, 1988a]. Furthermore, Davies and Deloignore (1987) found that hydroxyl radicals on their own were insufficient to fragment BSA, and that fragmentation only occurred when both superoxide and molecular oxygen were present in addition to the hydroxyl radicals.

There are two possible sources of free radicals in the glucose-induced protein fragmentation, the autoxidation of either glucose or the Amadori product. The idea that the radicals originate from the autoxidation of glucose is suggested by Hunt et al (1988a). He observes a small number of specific fragments and it is therefore likely that some further factor is controlling the specificity of this reaction. It is unlikely that this specificity is due to the production of radicals at copper binding sites because work done by Marx and Chevion (1985) using an ascorbate/copper system, where the specificity of the reaction was due to the copper binding at sites on BSA, produced fragments with different masses from those obtained by Hunt. The second possible source of free radicals in the glucose-mediated fragmentation is from the autoxidation of Amadori product. Fragmentation of BSA incubated with mixtures of copper and model Amadori products was observed by Kawakishi et al (1990). If autoxidation of Amadori product is the source of free radicals, then a certain amount of site specific cleavage might be expected. Therefore this mechanism may go some way to explaining why I obtained bands on fragmentation of HSA, even though they were broad and diffuse.

The glucose induced fragmentation of HSA is not affected by bound lipid. The fragmentation observed in both forms of DSA (where the delipidated form is more heavily fragmented) points to the bound lipid playing no role other than holding the DSA in a more compact conformation in which it may be protected. This is slightly surprising as lipid bound to HSA reacts through a free radical mediated pathway to form fluorophores. Hunt

and Dean (1989) found that BSA fragmented in the presence of peroxidising lipids (in the form of vesicles). He also showed that fragmentation preceded aggregation induced by reactions of protein with the aldehydes that are secondary products of lipid peroxidation. In Figure 3.4.12, no fragmentation of native HSA in the presence of high concentrations of iron is seen, but the formation of a small amount of high molecular mass substances is seen. It is possible that the lipids bound to HSA are already partially peroxidised [Fletcher and Tappel, 1971], so at high concentrations of iron they react further to produce aldehyde compounds such as MDA which are capable of inducing fluorescence and cross-linking, but not fragmentation [Hunt and Dean, 1989].

Preparations of HSA may include traces of proteolytic enzymes [Peters, 1985], but as preparations of HSA are heat-treated these enzymes could be denatured. If the enzymes are at all active in these incubations, then the cleavage of protein by traces of these enzymes does only occur when both copper and glucose are present. It was observed by Wolff and Dean (1986) that radical damage to proteins results in increased susceptibility of BSA to enzymic hydrolysis by both pepsin and trypsin. It has also been shown by Davies et al (1987b), that there is a direct relationship between oxidative denaturation of proteins and their increased susceptibility to proteolysis. However, Fong et al (1987), working with apoprotein B, found no inhibition of protein fragmentation when proteolytic inhibitors were included in the incubation, or when the reactants were heat-treated prior to incubation, suggesting that the mechanism of protein fragmentation did not involve proteases.

Further evidence that the cleavage of the protein backbone need not be mediated by proteases is the observation that the amount of carbonyl groups increase and yet there is no corresponding increase in amino groups when proteins are fragmented under oxidative conditions [Davies and Deloignore, 1987]. If cleavage of the peptide bond was occurring, then equal amounts of these groups would be expected. He suggests a mechanism for fragmentation where a proton is abstracted from the α -carbon to form an α -carbon radical, which in turn reacts with O_2 to produce a peroxy species. The decomposition of this carbon peroxide leads to fragmentation between the α -carbon and the carbonyl carbon so

that the peptide bond remains intact. A modification of this reaction which explains the specificity of radical mediated cleavage of BSA, is that the protein is cleaved at proline residues which are attacked by hydroxyl and superoxide radicals [Schuessler and Schilling, 1984; Wolff and Dean, 1986]. The masses of the fragments obtained by Schuessler and Schilling agreed well with the expected masses of fragments derived from cleavage at proline residues. Following *in vitro* glycation, both site-specific (occurring at a proline residue) and random fragmentation of the enzyme Cu,Zn-superoxide dismutase were observed by Ookawara et al (1992). Using ESR spectroscopy, they were able to show that reactive oxygen species, formed by the Maillard reaction, were involved in both fragmentations. Furthermore, they showed that the random fragmentation proceeds via a Fenton type reaction.

4.10.3 Conclusions

Both fluorophore formation and protein fragmentation are dependent on glucose and transition metal catalysts, showing that they are both glycoxidative reactions. Theoretically all reactions between organic compounds and molecular oxygen are not only metal catalysed, but are also free radical mediated processes. From this thesis, it is clear that protein fragmentation and the lipid component of fluorophore formation are indeed free radical mediated reactions. However, the role of free radicals in the lipid-independent component of glycofluorophore formation is less clear. The absence of any inhibitory effect by HEPES could be because free radicals produced at metal binding sites act at the site of their production, and are therefore not scavenged by HEPES.

As both fragmentation and the lipid-component of fluorophore formation are inhibited by the use of HEPES buffer, it is likely that these radical reactions occur at the surface of the protein where the radical produced can easily be scavenged. In fragmentation, more than one radical species may be involved, and bound lipids are on the surface of the protein making the radicals of both these reactions vulnerable to scavenging.

The probable role of 3-deoxyglucosone as an early intermediate in fluorophore formation does not exclude the possibility that a later reaction in the sequence involves free radicals. Indeed, as no fluorescence is seen without metal-catalysed oxidation, it is very likely that free radicals are involved in one of the steps of this reaction sequence.

Preincubation studies showed that glucose autoxidation products can mediate the formation of fluorophores, however, I was unable to determine whether or not this was a major pathway.

4.10.4 Future work

Future work on this project could include the direct incubation of proteins with 3-deoxyglucosone with different inhibitors, in order to determine if much of what has been observed is due to an intermediate such as 3-deoxyglucosone. This may also help to determine how dependent the reactions here are on glucose autoxidation.

Examination of cross-linking of HSA induced by glucose, 3-deoxyglucosone, MDA or lipid would be a natural extension from the observation of oligomers of native HSA when incubated with high concentrations of iron. Further work along this line could include incubating HSA with collagen (or other structural proteins) in the presence of glucose, 3-deoxyglucosone, MDA or lipid and seeing if adducts were formed between the two types of protein. This could contribute toward a model for the formation of diabetic complications such as the alteration in the filtration properties of the glomerular basement membrane.

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